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(54) Title: PORCINE CIRCOVIRUS RECOMBINANT POXVIRUS VACCINE

(57) Abstract: What is described is a recombinant poxvirus, such as avipox virus, containing foreign DNA from porcine circovirus 2. What are also described are immunological compositions containing the recombinant poxvirus for inducing an immunological response in a host animal to which the immunological composition is administered. Also described are methods of treating or preventing disease caused by porcine circovirus 2 by administering the immunological compositions of the invention to an animal in need of treatment or susceptible to infection by porcine circovirus 2.

TITLE OF THE INVENTION

Porcine Circovirus Recombinant Poxvirus Vaccine

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. application Serial No. 60/138,478, 5 filed June 10, 1999 and from the U.S. utility application filed May 31, 2000. Reference is made to WO-A-99 18214, 1998, French applications Nos. 97/12382, 98/00873, 98/03707, filed October 3, 1997, January 22, 1998, and March 20, 1998, and WO99/29717. Each of the aforementioned U.S., PCT and French applications, and each document cited in the text and the record or prosecution of each of the 10 aforementioned U.S., PCT and French applications ("application cited documents") and each document referenced or cited in each of the application cited documents, is hereby incorporated herein by reference; and, technology in each of the aforementioned U.S., PCT and French applications, and each document cited in the text and the record or prosecution of each of the aforementioned U.S., PCT and 15 French applications can be used in the practice of this invention.

Several publications are referenced in this application. Full citation to these documents is found at the end of the specification preceding the claims, and/or where the document is cited. These documents pertain to the field of this invention; and, each of the documents cited or referenced in this application ("herein cited 20 documents") and each document cited or referenced in herein cited documents are hereby incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to vectors, such as recombinant vectors; for instance, recombinant viruses, such as poxviruses, e.g., modified poxviruses and to 25 methods of making and using the same. In some embodiments, the invention relates to recombinant avipox viruses, such as canarypox viruses, e.g., ALVAC. The invention further relates to such vectors, e.g., poxviruses, that express gene products, e.g., antigen(s), ORF(s), and/or epitope(s) of interest therefrom, of porcine circovirus 2 (PCV2); to immunological compositions or vaccines. The invention yet further 30 relates to such vectors, e.g., poxviruses, that induce an immune response directed to or against PCV2 gene products and/or PCV2; and, to advantageously, such compositions that are immunological, immunogenic or vaccine compositions and/or confer protective immunity against infection by PCV2. The invention yet further relates to

the uses of and methods for making and using such vectors and compositions, as well as intermediates thereof, and said intermediates. And, the invention relates to the products therefrom, e.g., from the uses and methods involving the inventive recombinant or poxvirus, such as antibodies from expression.

5 **BACKGROUND OF THE INVENTION**

Postweaning multisystemic wasting syndrome (PMWS) is a recently recognized disease of young pigs. PMWS is characterized clinically by progressive weight loss and other symptoms such as tachypnea, dyspnea and jaundice. Pathologically, lymphocytic and granulomatous infiltrates, lymphadenopathy, and, 10 more rarely, lymphocytic and granulomatous hepatitis and nephritis have been observed (Clark, 1997; Harding, 1997).

This disease has been described in different European countries as well as in North America. Treatment and prevention of this disease are not currently available.

Several lines of evidence point to porcine circovirus as the etiologic agent of 15 PMWS (Ellis et al., 1998). Circoviruses have been recovered from pigs with PMWS, and antibodies to porcine circovirus have been demonstrated in pigs with the disease.

Circoviruses are single stranded circular DNA viruses found in a range of animal and plant species. Porcine circovirus was originally isolated as a contaminant from a continuous pig kidney cell line. The cell culture isolate has been designated 20 PK-15 (Meehan et al., 1997). More recently, porcine circovirus obtained from pigs with PMWS has been compared to PK-15. Such viruses differ substantially from PK-15 at the nucleotide and protein sequence level, and have been designated PCV2 (Meehan et al., 1998; Hamel et al., 1998).

As many as thirteen open reading frames (ORFs) have been identified in the 25 PCV2 genome (COL1 to COL13 in the French patent application 98 03707). Four of these ORFs share substantial homology with analogous ORFs within the genome of PK-15. ORF1 (Meehan et al., 1998; corresponding to COL4 in the French patent application 98 03707), comprising nt 398-1342 (GenBank accession number AF055392), has the potential to encode a protein with a predicted molecular weight of 30 37.7 kD. ORF2 (Meehan et al., 1998; corresponding to COL13 in the French patent application 98 03707), comprising nt 1381-1768 joined to 1-314 (GenBank accession number AF055392), may encode a protein with a predicted molecular weight of 27.8 kD. ORF3 (Meehan et al., 1998; corresponding to COL7 in the French patent

application 98 03707), comprising nt 1018-704 (GenBank accession number AF055392), may encode a protein with a predicted molecular weight of 11.9 kD. ORF4 (Meehan et al., 1998; corresponding to COL10 in the French patent application 98 03707), comprising nt 912-733 (GenBank accession number AF055392), may 5 encode a protein with a predicted molecular weight of 6.5 kD.

ORF1 of PCV2 is highly homologous (86% identity) to the ORF1 of the PK-15 isolate (Meehan et al., 1998). The ORF1 protein of PK-15 has been partially characterized (Meehan et al., 1997 ; Mankertz et al., 1998a). It is known to be essential for virus replication, and is probably involved in the viral DNA replication.

10 Protein sequence identity between the respective ORF2s was lower (66% identity) than that of the ORF1s but each of the ORF2s shared a highly conserved basic N-terminal region, similar to that observed in the N-terminal region of the major structural protein of the avian circovirus chicken anemia virus (CAV) (Meehan et al., 1998). Recently, Mankertz et al. (1998b) has suggested that the ORF2 of the PK-15 15 isolate (designated ORF 1 in Mankertz et al., 1998b) codes for a capsid protein.

Greater differences were observed between the respective ORF3s and ORF4s of the PK-15 isolate and PCV2. In each case, there was a deletion of the C-terminal region of PCV2 ORF4 and ORF3 compared to the corresponding ORFs present in the genome of the PK-15 isolate. The highest protein sequence homology was observed at 20 the N-terminal regions of both ORF3 and ORF4 (Meehan et al., 1998).

The transcription analysis of the genome of PCV2 has not been published yet. Recent data obtained with the PK-15 isolate indicated that the ORF2 transcript is spliced (Mankertz et al., 1998b).

25 Vaccinia virus has been used successfully to immunize against smallpox, culminating in the worldwide eradication of smallpox in 1980. With the eradication of smallpox, a new role for poxviruses became important, that of a genetically engineered vector for the expression of foreign genes (Panicali and Paoletti, 1982; Paoletti et al., 1984). Genes encoding heterologous antigens have been expressed in vaccinia, often resulting in protective immunity against challenge by the 30 corresponding pathogen (reviewed in Tartaglia et al., 1990). A highly attenuated strain of vaccines, designated MVA, has also been used as a vector for poxvirus-based vaccines. Use of MVA is described in U.S. Patent No. 5,185,146.

Two additional vaccine vector systems involve the use of naturally host-restricted poxviruses, avipox viruses. Both fowlpoxvirus (FPV; Taylor et al. 1988a, b) and canarypoxvirus (CPV; Taylor et al., 1991 & 1992) have been engineered to express foreign gene products. Fowlpox virus (FPV) is the prototypic virus of the 5 Avipox genus of the Poxvirus family. The virus causes an economically important disease of poultry which has been well controlled since the 1920's by the use of live attenuated vaccines. Replication of the avipox viruses is limited to avian species (Matthews, 1982) and there are no reports in the literature of avipoxvirus causing a productive infection in any non-avian species including man. This host restriction 10 provides an inherent safety barrier to transmission of the virus to other species and makes use of avipoxvirus based vaccine vectors in veterinary and human applications 15 an attractive proposition.

FPV has been used advantageously as a vector expressing antigens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was 20 expressed in an FPV recombinant (Taylor et al., 1988c). After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor et al., 1988c). FPV recombinants expressing the surface 25 glycoproteins of Newcastle Disease Virus have also been developed (Taylor et al., 1990 ; Edbauer et al., 1990).

Other attenuated poxvirus vectors have been prepared by genetic modifications of wild type strains of virus. The NYVAC vector, derived by deletion of specific virulence and host-range genes from the Copenhagen strain of vaccinia (Tartaglia et al., 1992) has proven useful as a recombinant vector in eliciting a protective immune 25 response against an expressed foreign antigen.

Another engineered poxvirus vector is ALVAC, derived from canarypox virus. ALVAC does not productively replicate in non-avian hosts, a characteristic thought to improve its safety profile (Taylor et al., 1991 & 1992). Both ALVAC and NYVAC are BSL-1 vectors.

30 One approach to the development of a subunit PCV2 vaccine is the use of live viral vectors to express relevant PCV2 ORFs. Recombinant poxviruses can be constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus

described in U.S. Patent Nos. 4,769,330; 4,722,848; 4,603,112; 5,110,587; 5,174,993; 5,494,807; and 5,505,941, the disclosures of which are incorporated herein by reference. It can thus be appreciated that provision of a PCV2 recombinant poxvirus, and of compositions and products therefrom particularly ALVAC based PCV2 recombinants and compositions and products therefrom, especially such recombinants containing ORFs 1 and/or 2 of PCV2, and compositions and products therefrom would be a highly desirable advance over the current state of technology.

OBJECTS AND SUMMARY OF THE INVENTION

It is therefore an object of this invention to provide compositions and methods for treatment and prophylaxis of infection with PCV2. It is also an object to provide a means to treat or prevent PMWS.

In one aspect, the present invention relates to an antigenic, immunological, immunogenic, or vaccine composition or a therapeutic composition for inducing an antigenic, immunogenic or immunological response in a host animal inoculated with the composition. The composition advantageously includes a carrier or diluent and a recombinant virus, such as a recombinant poxvirus. The recombinant virus or poxvirus contains and expresses an exogenous nucleic acid molecule encoding an ORF, antigen, immunogen, or epitope of interest from PCV2, or a protein that elicits an immunological response against PCV2 or conditions caused by PCV2, such as PMWS. For instance, the recombinant virus can be a modified recombinant virus or poxvirus; for example, such a virus or poxvirus that has inactivated therein virus-encoded genetic functions, e.g., nonessential virus-encoded genetic functions, so that the recombinant virus has attenuated virulence and enhanced safety. And, the invention further provides the viruses used in the composition, as well as methods for making and uses of the composition and virus.

The virus used in the composition according to the present invention is advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus and more advantageously, ALVAC. The modified recombinant virus can include, e.g., within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein, e.g., derived from PCV2 ORFs, e.g., PCV2 ORF 1 and/or 2.

In yet another aspect, the present invention relates to an immunogenic composition containing a modified recombinant virus having inactivated nonessential

virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety. The modified recombinant virus includes, e.g., within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein (e.g., derived from PCV2 ORFs, especially ORFs 1 and/or 2)

5 wherein the composition, when administered to a host, is capable of inducing an immunological response specific to the antigen.

In a still further aspect, the present invention relates to a modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence, and wherein the modified recombinant virus further

10 contains DNA from a heterologous source, e.g., in a nonessential region of the virus genome. The DNA can code for PCV2 genes such as any or all of PCV2 ORF1, ORF2, ORF3, or ORF4 (Meehan et al., 1998), or epitope(s) of interest therefrom. The genetic functions can be inactivated by deleting an open reading frame encoding a virulence factor or by utilizing naturally host-restricted viruses. The virus used

15 according to the present invention is advantageously a poxvirus, e.g., a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus.

Advantageously, the open reading frame that is deleted from the poxvirus or virus genome is selected from the group consisting of J2R, B13R + B14R, A26L, A56R, C7L – K1L, and I4L (by the terminology reported in Goebel et al., 1990); and,

20 the combination thereof. In this respect, the open reading frame comprises a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range gene region or a large subunit, ribonucleotide reductase; or, the combination thereof.

A suitable modified Copenhagen strain of vaccinia virus is identified as

25 NYVAC (Tartaglia et al., 1992), or a vaccinia virus from which has been deleted J2R, B13R+B14R, A26L, A56R, C7L-K11 and I4L or a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range region, and a large subunit, ribonucleotide reductase (See also U.S. Patent No. 5,364,773, 5,494,807, and 5,762,938, with respect to NYVAC and vectors having

30 additional deletions or inactivations from those of NYVAC that are also useful in the practice of this invention).

Preferably, the poxvirus vector is an ALVAC or, a canarypox virus which was attenuated, for instance, through more than 200 serial passages on chick embryo

fibroblasts (Rentschler vaccine strain), a master seed therefrom was subjected to four successive plaque purifications under agar from which a plaque clone was amplified through five additional passages. (See also U.S. Patent Nos. 5,756,103 and 5,766,599 with respect to ALVAC and TROVAC (an attenuated fowlpox virus useful in the practice of this invention); and U.S. Patents Nos. 6,004,777, 5,990,091, 5,770,212, 6,033,904, 5,869,312, 5,382,425, and WO 95/30018, with respect to vectors that also can be used in the practice of this invention, such as vectors having enhanced expression, vectors having functions deleted therefrom and vectors useful with respect to porcine hosts (for instance, vectors useful with porcine hosts can include a poxvirus, including a vaccinia virus, an avipox virus, a canarypox virus, and a swinepox virus), as well as with respect to terms used and teachings herein such as "immunogenic composition", "immunological composition", "vaccine", and "epitope of interest", and dosages, routes of administration, formulations, adjuvants, and uses for recombinant viruses and expression products therefrom).

The invention in yet a further aspect relates to the product of expression of the inventive recombinant poxvirus and uses therefor, such as to form antigenic, immunological or vaccine compositions for treatment, prevention, diagnosis or testing; and, to DNA from the recombinant poxvirus which is useful in constructing DNA probes and PCR primers.

These and other embodiments are disclosed or are obvious from and encompassed by the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

A better understanding of the present invention will be had by referring to the accompanying drawings, incorporated herein by reference, in which:

- FIG. 1 (SEQ ID NO:1) shows the nucleotide sequence of a 3.7 kilobase pair fragment of ALVAC DNA containing the C6 open reading frame.
- FIG. 2 shows the map of pJP102 donor plasmid.
- FIG. 3 (SEQ ID NO:8) shows the nucleotide sequence of the 2.5 kilobase pair fragment from pJP102 donor plasmid from the *Kpn*I (position 653) to the *Sac*I (position 3166) restriction sites.
- FIG. 4 shows the map of pJP105 donor plasmid.
- FIG. 5 shows the map of pJP107 donor plasmid.

- FIG. 6 (SEQ ID NO:11) shows the nucleotide sequence of the 3.6 kilobase pair fragment from pJP107 donor plasmid from the *Kpn*I (position 653) to the *Sac*I (position 4255) restriction sites.

DETAILED DESCRIPTION

5 In one aspect, the present invention relates to a recombinant virus, such as a recombinant poxvirus, containing therein a DNA sequence from PCV2, e.g., in a non-essential region of the poxvirus genome. The poxvirus is advantageously an avipox virus, such as fowlpox virus, especially an attenuated fowlpox virus, or a canarypox virus, especially an attenuated canarypox virus, such as ALVAC.

10 According to the present invention, the recombinant poxvirus expresses gene products of the foreign PCV2 gene. Specific ORFs of PCV2 are inserted into the poxvirus vector, and the resulting recombinant poxvirus is used to infect an animal. Expression in the animal of PCV2 gene products results in an immune response in the animal to PCV2. Thus, the recombinant poxvirus of the present invention may be 15 used in an immunological composition or vaccine to provide a means to induce an immune response which may, but need not be, protective.

20 The administration procedure for recombinant poxvirus-PCV2 or expression product thereof, compositions of the invention such as immunological, antigenic or vaccine compositions or therapeutic compositions, can be via a parenteral route (intradermal, intramuscular or subcutaneous). Such an administration enables a systemic immune response, or humoral or cell-mediated responses.

25 More generally, the inventive poxvirus- PCV2 recombinants, antigenic, immunological or vaccine poxvirus- PCV2 compositions or therapeutic compositions can be prepared in accordance with standard techniques well known to those skilled in the pharmaceutical or veterinary art. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical or veterinary arts taking into consideration such factors as the age, sex, weight, species and condition of the particular patient, and the route of administration. The compositions can be administered alone, or can be co-administered or sequentially administered 30 with compositions, e.g., with "other" immunological, antigenic or vaccine or therapeutic compositions thereby providing multivalent or "cocktail" or combination compositions of the invention and methods employing them. Again, the ingredients and manner (sequential or co-administration) of administration, as well as dosages can

be determined taking into consideration such factors as the age, sex, weight, species and condition of the particular patient, and, the route of administration. In this regard, reference is made to U.S. Patent No. 5,843,456, incorporated herein by reference, and directed to rabies compositions and combination compositions and uses thereof.

5 Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, vaginal, peroral, intragastric, etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration) such as sterile suspensions or emulsions. In such compositions the 10 recombinant poxvirus or antigens may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, adjuvants, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and 15 the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation. Suitable dosages can also be based upon the Examples below.

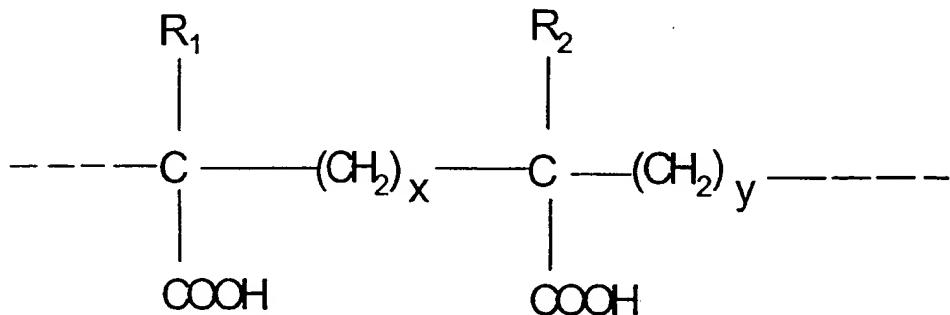
20 The compositions can contain at least one adjuvant compound chosen from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative.

The preferred adjuvant compounds are the polymers of acrylic or methacrylic acid which are cross-linked, especially with polyalkenyl ethers of sugars or 25 polyalcohols. These compounds are known by the term carbomer (Phameuropa Vol. 8, No. 2, June 1996). Persons skilled in the art can also refer to U.S. Patent No. 2,909,462 (incorporated herein by reference) which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms of at least three hydroxyls being 30 replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under the name

Carbopol® (BF Goodrich, Ohio, USA) are particularly appropriate. They are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among them, there may be mentioned Carbopol® 974P, 934P and 971P.

Among the copolymers of maleic anhydride and alkenyl derivative, the 5 copolymers EMA® (Monsanto) which are copolymers of maleic anhydride and ethylene, linear or cross-linked, for example cross-linked with divinyl ether, are preferred. Reference may be made to J. Fields et al., Nature, 186 : 778-780, 4 June 1960, incorporated herein by reference.

From the point of view of their structure, the polymers of acrylic or 10 methacrylic acid and the copolymers EMA® are preferably formed of basic units of the following formula :



15

in which :

20 - R_1 and R_2 , which are identical or different, represent H or CH_3 ,
 - $x = 0$ or 1, preferably $x = 1$
 - $y = 1$ or 2, with $x + y = 2$

For the copolymers EMA®, $x = 0$ and $y = 2$. For the carbomers, $x = y = 1$.

The dissolution of these polymers in water leads to an acid solution which will 25 be neutralized, preferably to physiological pH, in order to give the adjuvant solution into which the vaccine itself will be incorporated. The carboxyl groups of the polymer are then partly in COO^- form.

Preferably, a solution of adjuvant according to the invention, especially of carbomer, is prepared in distilled water, preferably in the presence of sodium chloride, the solution obtained being at acidic pH. This stock solution is diluted by adding it to the desired quantity (for obtaining the desired final concentration), or a substantial part thereof, of water charged with NaCl, preferably physiological saline (NaCl 9 g/l) all at once in several portions with concomitant or subsequent neutralization (pH 7.3 to 7.4), preferably with NaOH. This solution at physiological pH will be used as it is for mixing with the vaccine, which may be especially stored in freeze-dried, liquid or frozen form.

10 The polymer concentration in the final vaccine composition will be 0.01% to 2% w/v, more particularly 0.06 to 1% w/v, preferably 0.1 to 0.6% w/v.

15 The immunological compositions according to the invention may be associated to at least one live attenuated, inactivated, or sub-unit vaccine, or recombinant vaccine (e.g. poxvirus as vector or DNA plasmid) expressing at least one immunogen from another pig pathogen.

20 The invention encompasses vectors encoding and expressing equivalent nucleotide sequences, that is to say the sequences which change neither the functionality or the strain specificity (say strain of type 1 and strain of type 2) of the gene considered or those of the polypeptides encoded by this gene. The sequences differing through the degeneracy of the code are, of course, included.

25 The PCV-2 sequences used in the examples are derived from Meehan *et al.* (Strain Imp.1010 ; ORF1 nucleotides 398-1342; ORF2 nucleotides 1381-314; and correspond respectively to ORF4 and ORF13 in U.S. application Serial No. 09/161,092 of 25 September 1998 and to COL4 and COL13 in WO-A-9918214).

30 Other PCV-2 strains and their sequences have been published in WO-A-9918214 and are called Imp1008, Imp999, Imp1011-48285 and Imp1011-48121, as well as in A.L. Hamel *et al.* J. Virol. June 1998, vol 72, 6: 5262-5267 (GenBank AF027217) and in I. Morozov *et al.* J. Clinical Microb. Sept. 1998 vol. 36, 9: 2535-2541, as well as GenBank AF086834, AF086835 and AF086836, and give access to equivalent ORF sequences. These sequences, or ORFs therefrom, or regions thereof encoding an antigen or epitope of interest can also be used in the practice of this invention.

The invention also encompasses the equivalent sequences to those used herein and in documents cited herein; for instance, sequences that are capable of hybridizing

to the nucleotide sequence under high stringency conditions (see, e.g., Sambrook et al. (1989). Among the equivalent sequences, there may also be mentioned the gene fragments conserving the immunogenicity of the complete sequence, e.g., an epitope of interest.

5 The homology of the whole genome between PCV types 1 and 2 is about 75%. For ORF1, it is about 86%, and for ORF2, about 66%. On the contrary, homologies between genomes and between ORFs within type 2 are generally above 95%.

Also, equivalent sequences useful in the practice of this present invention, for ORF1, are those sequences having an homology equal or greater than 88%, 10 advantageously 90% or greater, preferably 92% or 95% or greater with ORF1 of strain Imp1010, and for ORF2, are those sequences having an homology equal or greater than 80%, advantageously 85% or greater, preferably 90% or 95% or greater with ORF2 of strain Imp1010.

15 ORF1 and ORF2 according to Meehan 1998 has the potential to encode proteins with predicted molecular weights of 37.7 kD and 27.8 kD respectively. ORF3 and ORF4 (according to Meehan et al. 1998, correspond to ORF7 and ORF10 respectively in WO-A-9918214) has the potential to encode proteins with predicted molecular weights of 11.9 and 6.5 kD respectively. The sequence of these ORFs is also available in Genbank AF 055392. They can also be incorporated in plasmids and 20 be used in accordance with the invention alone or in combination, e.g. with ORF1 and/or ORF2.

The other ORFs 1-3 and 5, 6, 8-9, 11-12 disclosed in U.S. application Serial No. 09/161,092 of 25 September 1998 (COLs 1-3 and 5, 6, 8-9, 11-12 in WO-A-9918214), or region(s) thereof encoding an antigen or epitope of interest, may be used 25 in the practice of this invention, e.g., alone or in combination or otherwise with each other or with the ORFs 1 and 2 or region(s) thereof encoding antigen(s) or epitope(s).

This invention also encompasses the use of equivalent sequences; for instance, from ORFs of various PCV-2 strains cited herein. For homology, one can determine that there are equivalent sequences which come from a PCV strain having an ORF2 and/or an ORF1 which have an homology as defined above with the corresponding 30 ORF of strain 1010.

For ORF3 according to Meehan, an equivalent sequence has homology thereto that is advantageously, for instance, equal or greater than 80%, for example 85% or

greater, preferably 90% or 95% or greater with ORF3 of strain Imp1010. For ORF4 according to Meehan 1998, advantageously an equivalent sequence has homology that is equal or greater than 86%, advantageously 90% or greater, preferably than 95% or greater with ORF4 of strain Imp1010.

5 From the genomic nucleotide sequence, e.g. those disclosed in WO-A-99 18214, it is routine art to determine the ORFs using a standard software, such as MacVector®. Also, alignment of genomes with that of strain 1010 and comparison with strain 1010 ORFs allows the one skilled in the art to readily determine the ORFs of the genome of another strain (e.g. other strains disclosed in WO-A-99 18214 or in 10 other herein cited documents).

Using software or making sequence alignment is not undue experimentation and provides direct access to equivalent ORFs or nucleic acid molecules.

Nucleotide sequence homology can be determined using the "Align" program of Myers and Miller, ("Optimal Alignments in Linear Space", CABIOS 4, 11-17, 15 1988, incorporated herein by reference) and available at NCBI. Alternatively or additionally, the term "homology" or "identity", for instance, with respect to a nucleotide or amino acid sequence, can indicate a quantitative measure of homology between two sequences. The percent sequence homology can be calculated as $(N_{ref} - N_{dif}) * 100 / N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two 20 sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence similarity of 75% with the sequence AATCAATC ($N_{ref} = 8$; $N_{dif} = 2$).

Alternatively or additionally, "homology" or "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman, 1983 PNAS USA 80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics TM Suite, Intelligenetics Inc. CA).. When RNA sequences are said to be similar, or

have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

RNA sequences within the scope of the invention can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

5 Additionally or alternatively, amino acid sequence similarity or identity or homology can be determined using the BlastP program (Altschul *et al.*, *Nucl. Acids Res.* 25, 3389-3402, incorporated herein by reference) and available at NCBI. The following references (each incorporated herein by reference) provide algorithms for 10 comparing the relative identity or homology of amino acid residues of two proteins, and additionally or alternatively with respect to the foregoing, the teachings in these references can be used for determining percent homology or identity: Needleman SB and Wunsch CD, "A general method applicable to the search for similarities in the amino acid sequences of two proteins," *J. Mol. Biol.* 48:444-453 (1970); Smith TF 15 and Waterman MS, "Comparison of Bio-sequences," Advances in Applied Mathematics 2:482-489 (1981); Smith TF, Waterman MS and Sadler JR, "Statistical characterization of nucleic acid sequence functional domains," Nucleic Acids Res., 11:2205-2220 (1983); Feng DF and Dolittle RF, "Progressive sequence alignment as a prerequisite to correct phylogenetic trees," *J. of Molec. Evol.*, 25:351-360 (1987); 20 Higgins DG and Sharp PM, "Fast and sensitive multiple sequence alignment on a microcomputer," CABIOS, 5: 151-153 (1989); Thompson JD, Higgins DG and Gibson TJ, "ClusterW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, positions-specific gap penalties and weight matrix choice, Nucleic Acid Res., 22:4673-480 (1994); and, Devereux J, Haeberlie P 25 and Smithies O, "A comprehensive set of sequence analysis program for the VAX," Nucl. Acids Res., 12: 387-395 (1984).

This invention not only allows for administration to adult pigs, but also to the young and to gestating females; in the latter case, this makes it possible, in particular, to confer passive immunity onto the newborns (maternal antibodies). Preferably, 30 female pigs are inoculated prior to breeding; and/or prior to serving, and/or during gestation. Advantageously, at least one inoculation is done before serving and it is preferably followed by an inoculation to be performed during gestation, e.g., at about mid-gestation (at about 6-8 weeks of gestation) and/or at the end of gestation (at about

J1304. Primer JP760 (SEQ ID NO:6) contains the 3' end of the H6 promoter from *EcoRV* and the 5' end of PCV2 ORF 2. Primer JP773 (SEQ ID NO:7) contains the 3' end of PCV2 ORF 2 followed by a *SalI* site. The product of PCR J1304 was then digested with *EcoRV/SalI* and cloned as a ~750 bp fragment into a ~4.5 kb

5 *EcoRV/SalI* fragment from pJP099 (see above in Example 1). The resulting plasmid was confirmed by sequence analysis and designated pJP102 (see the map of pJP102 in Figure 2 and the sequence (SEQ ID NO:8) in Figure 3). The sequence of ORF 2 matches sequence available in GenBank, Accession Number AF055392. The donor plasmid pJP102 (linearized with *NotI*) was used in an *in vitro* recombination (IVR)

10 test to generate ALVAC recombinant vCP1614 (see Example 6).

Sequence of the primers:

JP760 (SEQ ID NO:6)
CAT-CAT-CAT-GAT-ATC-CGT-TAA-GTT-TGT-ATC-GTA-ATG-ACG-TAT-
CCA-AGG-AGG-CG

15 JP773 (SEQ ID NO:7)
TAC-TAC-TAC-GTC-GAC-TTA-GGG-TTT-AAG-TGG-GGG-GTC

Example 3 - **CONSTRUCTION OF AN
ALVAC DONOR PLASMID
FOR PCV2 ORF2 AND ORF1**

20 PCV2 ORF 1 was amplified by PCR using primers JP774 (SEQ ID NO:9) and JP775 (SEQ ID NO:10) on plasmid pGem7Z-*Imp*1010-Sto_n-*EcoRI* No. 14 resulting in PCR J1311. Primer JP774 (SEQ ID NO:9) contains the 3' end of the H6 promoter from *NruI* and the 5' end of PCV2 ORF1. Primer JP775 (SEQ ID NO:10) contains the 3' end of PCV2 ORF1 followed by a *SalI* site. The product of PCR J1311 (~1 Kb)

25 was cloned into pCR2.1 (Invitrogen, Carlsbad, CA). The resulting plasmid was confirmed by sequence analysis and designated pJP104. The sequence of ORF1 matches sequence available in GenBank, Accession Number AF055392. A ~970 bp *NruI/SalI* fragment was isolated from pJP104 and cloned into a ~4.5 kb *NruI/SalI* fragment from pJP099 (see Example 1), resulting in a plasmid which was confirmed

30 by restriction analysis and designated pJP105 (see Figure 4). The donor plasmid pJP105 could be used in an *in vitro* recombination test (described in Example 6) to generate ALVAC recombinant expressing the PCV2 ORF1.

A ~838bp *BamHI/SalI* from pJP102 (see Example 2) was blunted using the Klenow fragment of DNA polymerase, and was cloned into the Klenow-blunted

11-13 weeks of gestation). Thus, an advantageous regimen is an inoculation before mating and/or serving and a booster inoculation during gestation. Thereafter, there can be reinoculation before each serving and/or during gestation at about mid-gestation and/or at the end of gestation. Preferably, reinoculations are during gestation. Male 5 pigs also can be inoculated, e.g., prior to mating.

Piglets, such as piglets from vaccinated females (e.g., inoculated as herein discussed), are inoculated within the first weeks of life, e.g., inoculation at one and/or two and/or three and/or four and/or five weeks of life. Preferably, piglets are first inoculated within the first week of life or within the third week of life (e.g., at the time 10 of weaning). Advantageously, such piglets are then boosted two to four weeks later.

The present invention is additionally described by the following illustrative, non-limiting Examples.

EXAMPLES

The invention in a preferred embodiment is directed to recombinant 15 poxviruses containing therein a DNA sequence from PCV2 in a nonessential region of the poxvirus genome. The recombinant poxviruses express gene products of the foreign PCV2 gene. In particular, ORF2 and ORF1 genes encoding PCV2 proteins were isolated, characterized and inserted into ALVAC (canarypox vector) recombinants. The molecular biology techniques used are the ones described by 20 Sambrook et al. (1989).

Cell Lines and Virus Strains. The strain of PCV2 designated Imp.1010-Stoan has been previously described (Meehan et al., 1998). It was isolated from mesenteric lymph node tissues from a diseased pig originating from Canada. Cloning of the PCV2 genome was described by Meehan et al. (1998). Plasmid pGem7Z-Imp1010- 25 Stoan-EcoRI No. 14 contains the PCV2 genome as an *Eco*RI fragment inserted into the *Eco*RI site of plasmid pGem-7Z (Promega, Madison, WI). The complete nucleotide sequence of the Imp.1010-Stoan PCV2 strain has been determined by Meehan et al. (1998) and is available under the GenBank accession number AF055392.

30 The parental canarypox virus (Rentschler strain) is a vaccinal strain for canaries. The vaccine strain was obtained from a wild type isolate and attenuated through more than 200 serial passages on chick embryo fibroblasts. A master viral seed was subjected to four successive plaque purifications under agar and one plaque

clone was amplified through five additional passages after which the stock virus was used as the parental virus in *in vitro* recombination tests. The plaque purified canarypox isolate is designated ALVAC. ALVAC was deposited November 14, 1996 under the terms of the Budapest Treaty at the American Type Culture Collection, 5 ATCC accession number VR-2547.

The generation of poxvirus recombinants involves different steps: (1) construction of an insertion plasmid containing sequences ("arms") flanking the insertion locus within the poxvirus genome, and multiple cloning site (MCS) localized between the two flanking arms (e.g., see Example 1); (2) construction of donor 10 plasmids consisting of an insertion plasmid into the MCS of which a foreign gene expression cassette has been inserted (e.g. see Examples 2 to 5); (3) *in vitro* recombination in cell culture between the arms of the donor plasmid and the genome of the parental poxvirus allowing the insertion of the foreign gene expression cassette into the appropriate locus of the poxvirus genome, and plaque purification of the 15 recombinant virus (e.g. see Example 6).

PCV2 recombinant immunogens may be used in association with PCV1 immunogens, for immunization of animals against PMWS. In a least preferred approach, PCV1 immunogens may be used without PCV2 immunogens.

Example 1 - CONSTRUCTION OF CANARYPOX
INSERTION PLASMID AT C6 LOCUS

Figure 1 (SEQ ID NO:1) is the sequence of a 3.7 kb segment of canarypox DNA. Analysis of the sequence revealed an ORF designated C6L initiated at position 377 and terminated at position 2254. The following describes a C6 insertion plasmid constructed by deleting the C6 ORF and replacing it with a multiple cloning site 25 (MCS) flanked by transcriptional and translational termination signals. A 380 bp PCR fragment was amplified from genomic canarypox DNA using oligonucleotide primers C6A1 (SEQ ID NO:2) and C6B1 (SEQ ID NO:3). A 1155 bp PCR fragment was amplified from genomic canarypox DNA using oligonucleotide primers C6C1 (SEQ ID NO:4) and C6D1 (SEQ ID NO:5). The 380 bp and 1155 bp fragments were fused 30 together by adding them together as template and amplifying a 1613 bp PCR fragment using oligonucleotide primers C6A1 (SEQ ID NO:2) and C6D1 (SEQ ID NO:5). This fragment was digested with *Sac*I and *Kpn*I, and ligated into pBluescript SK+ (Stratagene, La Jolla, CA, USA) digested with *Sac*I/*Kpn*I. The resulting plasmid,

pC6L was confirmed by DNA sequence analysis. It consists of 370 bp of canarypox DNA upstream of C6 ("C6 left arm"), vaccinia early termination signal, translation stop codons in six reading frames, an MCS containing *Sma*I, *Pst*I, *Xho*I and *Eco*RI sites, vaccinia early termination signal, translation stop codons in six reading frames and 1156 bp of downstream canary pox sequence ("C6 right arm").

5 Plasmid pJP099 was derived from pC6L by ligating a cassette containing the vaccinia H6 promoter (described in Taylor et al. (1988c), Guo et al. (1989), and Perkus et al. (1989)) coupled to a foreign gene into the *Sma*I/*Eco*RI sites of pC6L. This plasmid pJP099 contains a unique *Eco*RV site and a unique *Nru*I site located at 10 the 3' end of the H6 promoter, and a unique *Sa*II site located between the STOP codon of the foreign gene and the C6 left arm. The ~4.5 kb *Eco*RV/*Sa*II or *Nru*I/*Sa*II fragment from pJP099 contains therefore the plasmid sequence (pBluescript SK+ ; Stratagene, La Jolla, CA, USA), the 2 C6 arms and the 5' end of the H6 promoter until the *Eco*RV or *Nru*I site.

15 Sequences of the primers:

Primer C6A1 (SEQ ID NO:2)

ATCATCGAGCTCGCGGCCCTATCAAAAGTCTTAATGAGTT

Primer C6B1 (SEQ ID NO:3)

GAATTCTCGAGCTGCAGCCGGTTTTATAGCTAATTAGTCATTTTC

20 Primer C6C1 (SEQ ID NO:4)

CCCGGGCTGCAGCTCGAGGAATTCTTTATTGATTAAGTCAAATGAG

TATATATAATTGAAAAAGTAA

Primer C6D1 (SEQ ID NO:5)

25 GATGATGGTACCTTCATAAATACAAGTTGATTAAACTTAAGTTG

Example 2 - **CONSTRUCTION OF ALVAC
DONOR PLASMID FOR PCV2 ORF2**

Plasmid pGem7Z-Imp1010-Stoorn-EcoRI No. 14, containing the PCV2 genome as an *Eco*RI fragment in plasmid pGem-7Z, was digested with *Eco*RI, and a 30 1768bp fragment was isolated and ligated.

In order to insert PCV2 ORF 2 into an appropriate ALVAC insertion vector: Primers JP760 (SEQ ID NO:6) and JP773 (SEQ ID NO:7) were used to amplify PCV2 ORF 2 from the 1768bp ligated *Eco*RI fragment (see above) resulting in PCR

EcoRI site of pJP105. Clones were checked for orientation of insert by restriction analysis and a head-to-head orientation was chosen. This plasmid was confirmed by sequence analysis and designated pJP107 (see the map of pJP107 in Figure 5 and the sequence (SEQ ID NO:11) in Figure 6). The donor plasmid pJP107 (linearized with 5 *NotI*) was used in an *in vitro* recombination 5 (IVR) test to generate the ALVAC recombinant vCP1615 (see Example 6).

Sequence of the primers:

JP774 (SEQ ID NO:9)

CAT-CAT-CAT-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT-CGT-AAT-GCC-

10 CAG-CAA-GAA-GAA-TGG

JP775 (SEQ ID NO:10)

TAC-TAC-TAC-GTC-GAC-TCA-GTA-ATT-TAT-TTC-ATA-TGG

Example 4 - CONSTRUCTION OF ALVAC
DONOR PLASMID FOR PCV1 ORF2

15 Plasmid pPCV1 (B. Meehan *et al.* J. Gen. Virol. 1997. 78. 221-227), containing the PCV1 genome as a *PstI* fragment in plasmid pGem-7Z, was used as a template to amplify the PCV1 ORF2.

In order to insert PCV2 ORF 2 into an appropriate ALVAC insertion vector :

Primers JP787 (SEQ ID NO:12) and JP788 (SEQ ID NO:13) were used to amplify

20 PCV1 ORF 2 from plasmid pPCV1 (see above) resulting in PCR J1315. Primer JP787 (SEQ ID NO:12) contains the 3' end of the H6 promoter from *EcoRV* and ORF 2 followed by a *SaI* site. The product of PCR J1315 was then digested with *EcoRV/SaI* and cloned as a ~750 bp fragment into a ~4.5 kb *EcoRV/SaI* fragment from pJP099 (see above in Example 1). The resulting plasmid was confirmed by 25 sequence analysis and designated pJP113. The sequence of ORF 2 matches sequence available in GenBank, Accession Number U49186. The donor plasmid pJP113 (linearized with *NotI*) was used in an *in vitro* recombination (IVR) test to generate ALVAC recombinant vCP1621 (see Example 7).

Sequence of the primers:

30 JP787 (SEQ ID NO:12)

CAT-CAT-CAT-GAT-ATC-CGT-TAA-GTT-TGT-ATC-GTA-ATG-ACG-TGG-

CCA-AGG-AGG-CG

JP788 (SEQ ID NO:13)

TAC-TAC-TAC-GTC-GAC-TTA-TTT-ATT-TAG-AGG-GTC-TTT-TAG-G

**Example 5 - CONSTRUCTION OF AN
ALVAC DONOR PLASMID
FOR PCV1 ORF2 AND ORF1**

5 Plasmid pPCV1 (see Example 4 above), containing the PCV1 genome as a *Pst*I fragment in plasmid pGem-7Z, was digested with *Pst*I, and a 1759 bp fragment was isolated and ligated.

Primers JP789 (SEQ ID NO:14) and JP790 (SEQ ID NO:15) were used to amplify PCV1 ORF1 from the 1759 bp ligated *Pst*I fragment (see above), resulting in 10 PCR J1316. Primer JP789 (SEQ ID NO:14) contains the 3' end of the H6 promoter from *Nru*I and the 5' end of PCV1 ORF1. Primer JP790 (SEQ ID NO:15) contains the 3' end of PCV1 ORF1 followed by a *Sa*II site. The product of PCR J1316 (~1 Kb) was cloned into pCR2.1 (Invitrogen, Carlsbad, CA). The resulting plasmid was confirmed by sequence analysis and designated pJP114. The sequence of ORF1 15 matches sequence available in GenBank, Accession Number U49186. A ~970 bp *Nru*I/*Sa*II fragment was isolated from pJP114 and cloned into a ~4.5 kb *Nru*I/*Sa*II fragment from pJP099 (see Example 1), resulting in a plasmid which was confirmed by restriction analysis and designated pJP115. The donor plasmid pJP115 could be used in an *in vitro* recombination test (described in Example 7) to generate ALVAC 20 recombinant expressing the PCV1 ORF1.

A ~838bp *Bam*HI/*Sa*II from pJP113 (see Example 4) was blunted using the Klenow fragment of DNA polymerase, and was cloned into the Klenow-blunted *Eco*RI site of pJP115. Clones were checked for orientation of insert by restriction analysis and a head-to-head orientation was chosen. This plasmid was confirmed by 25 sequence analysis and designated pJP117. The donor plasmid pJP117 (linearized with *Not*I) was used in an *in vitro* recombination (IVR) test to generate the ALVAC recombinant vCP1622 (see Example 7).

Sequence of the primers:

JP789 (SEQ ID NO:14)

30 CAT-CAT-CAT-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT-CGT-AAT-GCC-
AAG-CAA-GAA-AAG-CGG

JP790 (SEQ ID NO:15)

TAC-TAC-TAC-GTC-GAC-TCA-GTA-ATT-TAT-TTT-ATA-TGG

Example 6 - GENERATION OF ALVAC-PCV2 RECOMBINANTS

Plasmids pJP102 (see Example 2 and Figure 2) and pJP107 (see Example 3 and Figure 5) were linearized with *No*I and transfected into ALVAC infected primary CEF cells by using the calcium phosphate precipitation method previously described 5 (Panicali and Paoletti, 1982 ; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to specific PCV2 radiolabeled probes and subjected to four sequential rounds of plaque purification until a pure population was achieved. One representative plaque from each IVR was then amplified and the resulting ALVAC recombinants were designated vCP1614 and vCP1615. The vCP1614 virus is the 10 result of recombination events between ALVAC and the donor plasmid pJP102, and it contains the PCV2 ORF2 inserted into the ALVAC C6 locus. The vCP1615 virus is the result of recombination events between ALVAC and the donor plasmid pJP107, and it contains the PCV2 ORF2 and ORF1 inserted into the ALVAC C6 locus in a head-to-head orientation.

15 In a similar fashion, a recombinant ALVAC expressing only PCV2 ORF1 can be generated using the donor plasmid pJP105 described in Example 3.

Immunofluorescence. In order to determine if the PCV2 proteins were expressed in ALVAC recombinant infected Vero cells, immunofluorescence (IF) analysis was performed. Infected Vero cells were washed with PBS 24 hrs after 20 infection (m.o.i. of approx. 10) and fixed with 95% cold acetone for 3 minutes at room temperature. Five monoclonal antibody (MAb) preparations (hybridoma supernatant) specific for PCV2 ORF1 (PCV2 199 1D3GA & PCV2 210 7G5GD) or ORF2 (PCV2 190 4C7CF, PCV2 190 2B1BC & PCV2 190 3A8BC) were used as the first antibody. These specific monoclonal antibodies were obtained from Merial-Lyon. Monoclonal 25 antibodies can also be obtained following the teachings of documents cited herein, e.g. WO-A-99 18214, 1998, French applications Nos. 97/12382, 98/00873, 98/03707, filed October 3, 1997, January 22, 1998, and March 20, 1998, and WO99/29717, incorporated herein by reference. The IF reaction was performed as described by Taylor et al. (1990).

30 PCV2 specific immunofluorescence with the three ORF2-specific antibodies could be detected in cells infected with vCP1614 and cells infected with vCP1615. PCV2 specific immunofluorescence with the two ORF1-specific antibodies could be detected in cells infected with vCP1615 only. These results indicated that, as

expected, vCP1614 expresses only ORF2, whereas vCP1615 expresses both ORF1 and ORF2. No fluorescence was detected in parental ALVAC infected Vero cells, nor in uninfected Vero cells.

Example 7 - GENERATION OF ALVAC-PCV1 RECOMBINANTS

5 Plasmids pJP113 (see Example 4) and pJP117 (see Example 5) were linearized with *Not*I and transfected into ALVAC infected primary CEF cells by using the calcium phosphate precipitation method previously described (Panicali and Paoletti, 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to specific PCV1 radiolabeled probes and subjected to four sequential 10 rounds of plaque purification until a pure population was achieved. One representative plaque from each IVR was then amplified and the resulting ALVAC recombinants were designated vCP1621 and vCP1622. The vCP1621 virus is the result of recombination events between ALVAC and the donor plasmid pJP113, and it contains the PCV1 ORF2 inserted into the ALVAC C6 locus. The vCP1622 virus is the result 15 of recombination events between ALVAC and the donor plasmid pJP117, and it contains the PCV1 ORF2 and ORF1 inserted into the ALVAC C6 locus in a head-to-head orientation.

In a similar fashion, a recombinant ALVAC expressing only PCV1 ORF1 can be generated using the donor plasmid pJP115 described in Example 5.

20 Immunofluorescence. In order to determine if the PCV1 proteins were expressed in ALVAC recombinant infected Vero cells, immunofluorescence (IF) analysis was performed. Infected Vero cells were washed with PBS 24 hrs after infection (m.o.i. of approx. 10) and fixed with 95% cold acetone for 3 minutes at room temperature. A specific anti-PCV1 pig polyclonal serum (Allan G. et al. Vet. 25 Microbiol. 1999. 66: 115-123) was used as the first antibody. The IF reaction was performed as described by Taylor et al. (1990).

PCV1 specific immunofluorescence could be detected in cells infected with vCP1621 and cells infected with vCP1622. These results indicated that, as expected, vCP1621 and vCP1622 express PCV1-specific products. No fluorescence was 30 detected with a PCV2-specific pig polyclonal serum in cells infected with vCP1621 and in cells infected with vCP1622. No fluorescence was detected in parental ALVAC infected Vero cells, nor in uninfected Vero cells.

Example 8 - **FORMULATION OF RECOMBINANT
CANARYPOX VIRUSES WITH CARBOPOL™ 974P**

For the preparation of vaccines, recombinant canarypox viruses vCP1614 and vCP1615 (Example 6) can be mixed with solutions of carbomer. In the same fashion, recombinant canarypox viruses vCP1621 and vCP1622 (Example 7) can be mixed with solutions of carbomer. The carbomer component used for vaccination of pigs according to the present invention is the Carbopol™ 974P manufactured by the company BF Goodrich (molecular weight of # 3,000,000). A 1.5 % Carbopol™ 974P stock solution is first prepared in distilled water containing 1 g/l of sodium chloride.

This stock solution is then used for manufacturing a 4 mg/ml Carbopol™ 974P solution in physiological water. The stock solution is mixed with the required volume of physiological water, either in one step or in several successive steps, adjusting the pH value at each step with a 1N (or more concentrated) sodium hydroxide solution to get a final pH value of 7.3-7.4. This final Carbopol™ 974P solution is a ready-to-use solution for reconstituting a lyophilized recombinant virus or for diluting a concentrated recombinant virus stock. For example, to get a final viral suspension containing 10^8 pfu per dose of 2 ml, one can dilute 0,1 ml of a 10^9 pfu/ml stock solution into 1,9 ml of the above Carbopol™ 974P 4 mg/ml ready-to-use solution. In the same fashion, Carbopol™ 974P 2 mg/ml ready-to-use solutions can also be prepared.

Example 9 - **IMMUNIZATION OF PIGS
AND SUBSEQUENT CHALLENGE**

9.1. IMMUNIZATION OF 1 DAY-OLD PIGLETS

Groups of piglets, caesarian-derived at Day 0, are placed into isolators. The piglets are vaccinated by intramuscular route at Day 2 with various vaccine solutions. Vaccine viral suspensions are prepared by dilution of recombinant viruses stocks in sterile physiological water (NaCl 0.9 %). Suitable ranges for viral suspensions can be determined empirically, but will generally range from 10^6 to 10^{10} , and preferably about 10^{10} , pfu/dose. Vaccine solutions can also be prepared by mixing the recombinant virus suspension with a solution of Carbopol™ 974P, as described in Example 8.

Piglets are vaccinated either with:

Recombinant virus vCP1614 (Example 2);

Recombinant virus vCP1615 (Example 3);

Recombinant virus vCP1614 mixed with Carbopol (4 mg/ml solution); or

Recombinant virus vCP1615 mixed with Carbopol (4 mg/ml solution).

The viral suspensions contain 10^8 plaque forming units (pfu) per dose. Each viral suspension is injected by intramuscular route under a volume of 1 ml. The 5 intramuscular injection is administered into the muscles of the neck.

Two injections of viral suspensions are administered at Day 2 and Day 14 of the experiment. A challenge is done on Day 21 by an oronasal administration of a viral suspension prepared from a culture of PCV-2 virulent strain. After challenge, piglets are monitored during 3 weeks for clinical signs specific of the post-weaning

10 multisystemic syndrome. The following signs are scored :

Rectal temperature: daily monitoring for 2 weeks post-challenge, then 2 measures of rectal temperature during the third week.

Weight: piglets are weighed right before the challenge, and then weekly during the first 3 weeks post-challenge.

15 Blood samples are taken at Day 2, day 14, Day 21, Day 28, Day 35 and Day 42 of the experiment in order to monitor viremia levels and anti-PCV-2 specific antibody titers.

Necropsies: at Day 42, all surviving piglets are humanely euthanized and necropsied to look for specific PWMS macroscopic lesions. Tissue samples are prepared from liver, lymph nodes, spleen, kidneys and thymus in order to look for specific

20 histological lesions.

9.2. IMMUNIZATION OF 5-7 WEEK-OLD PIGLETS

5-7 week-old piglets, free of anti-PCV-2 specific maternal antibodies, are vaccinated by intramuscular route with various vaccine solutions. Vaccine viral suspensions are prepared by dilution of recombinant viruses stocks in sterile

25 physiological water (NaCl 0.9 %). Vaccine solutions can also be prepared by mixing the recombinant virus suspension with a solution of CarbopolTM 974P, as described in Example 8.

Piglets are vaccinated either with:

Recombinant virus vCP1614 (Example 2);

30 Recombinant virus vCP1615 (Example 3);

Recombinant virus vCP1614 mixed with Carbopol (4 mg/ml solution); or

Recombinant virus vCP1615 mixed with Carbopol (4 mg/ml solution).

The viral suspensions contain 10^8 plaque forming units (pfu) per dose. Each viral suspension is injected by intramuscular route under a volume of 2 ml. The intramuscular injection is administered into the muscles of the neck.

5 Two injections of the viral suspensions are administered at Day 0 and Day 21 of the experiment. A challenge is done at Day 35 by an oronasal administration of a viral suspension prepared from a culture of PCV-2 virulent strain. After challenge, piglets are monitored during 8 weeks for clinical signs specific of the post-weaning multisystemic syndrome. The clinical monitoring is identical to the one described in Example 9.1. except that total duration of monitoring is 8 weeks instead of 3 weeks.

10 Necropsies are done throughout the experiment for piglets dying from the challenge and at the end of the experiment (Day 97) for all surviving piglets. Tissue samples are the same as described in Example 9.1.

9.3. IMMUNIZATION OF NEWBORN PIGLETS

Groups of 3 or 4 piglets, caesarian-delivered day 0 are placed into isolators.

15 Day 2 the piglets are vaccinated with 10^8 pfu of vCP1614, vCP1615 or parental ALVAC vector in 1 ml of PBS by intramuscular route on the side of the neck. A second injection of vaccine or placebo is administered at day 14. Vaccination with ALVAC recombinant is well tolerated by piglets and no evidence of adverse reaction to vaccination is noted. The piglets are challenged day 21 by oronasal administration 20 of a PCV-2 viral suspension, 1 ml in each nostril. Day 45 necropsies are performed and samples of tissues are collected for virus isolation.

Necropsy results:

- PMWS is characterized generally by lymphadenopathy and more rarely by hepatitis or nephritis. So the gross findings in lymph nodes are scored for each 25 piglet in the following manner : 0 = no visible enlargement of lymph nodes ; 1 = mild lymph nodes enlargement, restricted to bronchial lymph nodes ; 2 = moderate lymph nodes enlargement, restricted to bronchial lymph nodes ; 3 = severe lymph nodes enlargement, extended to bronchial, submandibular prescapular and inguinal lymph nodes.

<u>Groups</u>	<u>Scores</u>
vCP 1614	
	0.5
	0.0
	0.0
	1.0
mean	0.38
standard deviation	0.48
vCP 1615	
	0.0
	0.5
	0.5
	1.0
mean	0.5
standard deviation	0.41
Controls	
	2.0
	2.5
	2.5
	2.5
mean	2.38
standard deviation	0.25

Bronchial lymphadenopathy for PCV-2 is a prominent gross finding. A significant reduction of the lymph nodes lesion in relation to control group is observed after immunization with vCP 1614 and vCP 1615 ($p \leq 0.05$).

5

* * *

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

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WHAT IS CLAIMED IS:

1. A recombinant virus comprising DNA from porcine circovirus 2.
2. The recombinant virus of claim 1 which is a poxvirus.
3. The recombinant poxvirus of claim 2 which is an avipox virus.
- 5 4. The recombinant avipox virus of claim 3 which is ALVAC.
5. The recombinant ALVAC virus of claim 4, wherein the DNA from porcine circovirus 2 codes for and is expressed as the porcine circovirus major capsid protein or an epitope of interest.
6. The recombinant ALVAC virus of claim 4, wherein the DNA from porcine circovirus 2 comprises of the open reading frame 2 (ORF2) of porcine circovirus 2.
- 10 7. The recombinant ALVAC virus of claim 4, wherein the DNA from porcine circovirus 2 comprises of the open reading frame 1 (ORF1) of porcine circovirus 2.
- 15 8. The recombinant ALVAC virus of claim 4, wherein the DNA from porcine circovirus 2 comprises of the open reading frames 1 and 2 (ORF1 and 2) of porcine circovirus.
9. The recombinant ALVAC virus of claim 4 which is vCP1614 or vCP1615.
10. An immunological composition for inducing an immunological response in a host inoculated with the immunological composition, the immunological composition comprising a carrier and the recombinant virus of claim 1.
- 20 11. An immunological composition for inducing an immunological response in a host inoculated with the immunological composition, the immunological composition comprising a carrier and the recombinant virus of claim 5.
12. An immunological composition for inducing an immunological response in a host inoculated with the immunological composition, the immunological composition comprising a carrier and the recombinant virus of claim 6.
- 25 13. An immunological composition for inducing an immunological response in a host inoculated with the immunological composition, the immunological composition comprising a carrier and the recombinant virus of claim 7.
14. An immunological composition for inducing an immunological response in a host inoculated with the immunological composition, the immunological composition comprising a carrier and the recombinant virus of claim 8.

15. An immunological composition for inducing an immunological response in a host inoculated with the immunological composition, the immunological composition comprising a carrier and the recombinant virus of claim 9.
16. A method for inducing an immunological response in a host comprising administering to the host the immunological composition of claim 11.
- 5 17. A method for inducing an immunological response in a host comprising administering to the host the immunological composition of claim 12.
18. A method for inducing an immunological response in a host comprising administering to the host the immunological composition of claim 13.
- 10 19. A method for inducing an immunological response in a host comprising administering to the host the immunological composition of claim 14.
20. A method for inducing an immunological response in a host comprising administering to the host the immunological composition of claim 15.

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HindIII (1)

1 AAGCTTCTATCAAAAGCTTAATGAGTTAGGTGTAGATAGTATAGATATTACTACAAAGGTATTCAATT
 71 TCCTATCAATTCTAAAGTAGATGATATAATAACTCAAAAGATGATGATAGTAGATAATAGATAACGCTCAT
 141 ATAATGACTGAAATTGGACGGTCACATTAAATCATCACGGCTCATAGTTCAACTGCATAGATC
 211 AAAATCTCACTAAAAAGATAGCCGATGTATTCAGAGAGATGGACATCTAACTACGCTAAAGAAATTAC
 281 AGTTATAAATAATACATAATGGATTTGTTATCATCAGTTATTTAACATAAGTACAATAAAAGTATT
 351 AAATAAAAATACCTTACGAAAAATGTCATTACAAAATATAATTACAGAACATCTAGT
 1 Met Ser Leu Leu Glu Lys Leu Tyr Phe Thr Glu Glu Ser Ile Val
 421 AGAGTCCTTAAGAGTTATAATTAAAAGATAACCCATAATGTAATATTACACATCAGATGATGATACT
 151 Glu Ser Phe Lys Ser Tyr Asn Leu Lys Asp Asn His Asn Val Ile Phe Thr Thr Ser Asp Asp Asp Thr
 491 GTTGTAGTAATAAAATGAAGATAATGTAATGTTATCTACAAGATTATTATCATTTGATAAAAATCTGTTT
 391 Val Val Val Ile Asn Glu Asp Asn Val Leu Leu Ser Thr Arg Leu Leu Ser Phe Asp Lys Ile Leu Phe P
 561 TTAACCTCTTAATAACGGTTATCAAACGAACTATTAGTGATAACAATATTAGATATAGATACTCA
 621 He Asn Ser Phe Asn Asn Glu Leu Ser Lys Tyr Glu Thr Ile Ser Asp Thr Ile Leu Asp Ile Asp Thr His
 631 TAATTATTATACCTAGTTCTCTTCTTGTAGATATTCTAAAAAGAGCGCTGTGATTAGAAATT
 851 Asn Tyr Tyr Ile Pro Ser Ser Ser Leu Leu Asp Ile Leu Lys Lys Arg Ala Cys Asp Leu Glu Leu
 701 GAAGATCTAAATTATGCGTTAATAGGAGACAATAGTAACCTATATTATAAAAGATATGACTTACATGAATA
 1091 Glu Asp Leu Asn Tyr Ala Leu Ile Glu Asp Asn Ser Asn Leu Tyr Tyr Lys Asp Met Thr Tyr Met Asn A
 771 ATTGGTTATTACTAAAGGATTATTAGATTACAAGTTGTATTATTGCGCGATGTAGATAATGTTACAA
 1321 Asn Thr Leu Phe Thr Lys Glu Leu Leu Asp Tyr Lys Phe Val Leu Leu Arg Asp Val Asp Lys Cys Tyr Lys

NruI (880)

NdeI (901)

841 ACAGTATAATAAAAGAACTACTATAATAGATATAATACATCGCGATAACAGACAGTATAACATATGGGTT
 1551 Glu Tyr Asn Lys Asn Thr Ile Asp Ile His Arg Asp Asn Arg Glu Tyr Asn Ile Trp Val
 911 AAAATGTTATAGAATACTGTTCTCTGGCTATATAATTATGGTACATGATCTAAAGCCGCTGCTGAAG
 1791 Lys Asn Val Ile Glu Tyr Cys Ser Pro Gly Tyr Ile Leu Trp Leu His Asp Leu Lys Ala Ala Glu Val
 981 ATGATTGGTTAAAGATAACGATAACCGATAAAACGAAATTATCTGGGATAAAATTATACACTTCGAGTTCT
 2021 Asp Trp Leu Arg Tyr Asp Asn Arg Ile Asn Glu Leu Ser Ala Asp Lys Leu Tyr Thr Phe Glu Phe Ile
 1051 ACTTATAATTAGAAAATAATATAAAACATTACGAGTAGGTACATAATTGACATCCAAACAAGATAATA
 2251 Val Ile Leu Glu Asn Asn Ile Lys His Leu Arg Val Glu Ile Val His Pro Asn Lys Ile
 1121 CCTAATGGTACATCTAATAATATACTTACTGATTCTATCTTACGTAGAAGAACTAATATATCATCATA
 2491 Ala Asn Glu Thr Ser Asn Asn Ile Leu Thr Asp Phe Leu Ser Tyr Val Glu Leu Ile Tyr His His A

EcoRI (1223)

1191 ATTCATCTATAATATTGGCCGGATTTTTAGAATTCTTGTAGGACCCACTATTATCAGAATTATTTC
 2721 Asn Ser Ser Ile Leu Ala Glu Tyr Phe Leu Glu Phe Phe Glu Thr Thr Ile Leu Ser Glu Phe Ile Ser
 1261 TTTCATCTCTGAATGGGTAAATGAATAGTAACCTGTTAGTACACCTGAAACAGGGTATGAAGCTATACTC
 2951 Ser Ser Ser Glu Trp Val Met Asn Ser Asn Cys Leu Val His Leu Lys Thr Glu Tyr Glu Ala Ile Leu
 1331 TTGGATGCTAGTTATTCTTCAACTCTCTACTAAAAGCAATTATGAAAAATTGGACAAAGAAAACCTT
 3191 Phe Asp Ala Ser Leu Phe Phe Glu Leu Ser Thr Lys Ser Asn Tyr Val Lys Tyr Trp Thr Lys Lys Thr L
 1401 TGCAGTATAAGAACCTTTAAAGACGGTAAACAGTTAGGAAATATATAATTAAAGAAAGATAGTCAGGT
 3421 Glu Glu Tyr Lys Asn Phe Phe Lys Asp Glu Lys Glu Leu Ala Lys Tyr Ile Ile Lys Lys Asp Ser Glu Val

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1473 GATAGATAGAGTATGTTATTCACCGCAGCTGTATATAATCAGCTAACCTACTTAATGGATACGTTAAA
 365 IleAspArgValCysTyrLeuHisAlaAlaValTyrAsnHisValThrTyrLeuMetAspThrPheLys

 1541 ATTCCCTGGTTTGTATTAACTCCGGAATGATAGATATACTACTGTTGGAATATTGCATAAGGATA
 389 IleProGlyPheAspPheSerGlyMetIleAspIleLeuLeuPheGlyIleLeuHisLysAspA

 1611 ATGAGAAATATATTTATCCGAAACGTTCTGTAACTAATATAATCAGAACTATCTATGCAGATT
 412 snGluAsnIlePheTyrProLysArgValSerValThrAsnIleSerGluSerIleTyrAlaAspPh

 1681 TTACTTTATCAGATGTTAATAATTCACTAAAAGATAGAATAATAAACTAIGTTCTCTATACTCGCA
 435 IleTyrPhelIleSerAspValAsnLysPheSerLysLysIleGluTyrLysThrMetPheProIleLeuAla

 1751 GAAAACACTATCCAAAAGGAAGGCCCTATTTACACATACATCTAACGAAGATCTCTGTCTATCTGTT
 459 GluAsnTyrTyrProLysGlyArgProTyrPheThrHisThrSerAsnGluAspLeuSerIleCysL

 1821 TATGCGAAGTAAACAGTTGTAAAGATATAAAACCTATTATATTCTAAAAGGATATTCAGCAA
 482 IleCysGluValThrValCysLysAspIleLysAsnProLeuLeuTyrSerLysAspIleSerAlaL

 1891 ACCATTCAAGGTTTATTCAGATCTGCGATATAAAACGGCTGTTGAGTTAAGAGGATATAAAAGA
 505 ArgPheIleGlyLeuPheThrSerValAspIleAsnThrAlaValGluLeuArgGlyTyrLysIleArg

 1961 GTAATAGGATGTTAGAATGGCCTGAAAAGATAAAAATTTAACTCTAACATACATTAGATTAT
 529 ValIleGlyCysLeuGluTrpProGluLysIlePheAsnSerAsnProThrTyrIleArgLeuL

 2031 TACTAACAGAAAGACGTTAGATAATTCTACATTCCTATCTGCTTAAATTAACTATAACAGAGGATATAGC
 552 IleLeuThrGluArgArgLeuAspIleLeuHisSerTyrLeuLeuLysPheAsnIleThrGluAspIleAl

 2101 TACCAAGAGATGGAGTCAGAAATAATTACCTATAAATTCTTATCGTCAGTTATTGTAGATCGTATACT
 575 ThrArgAspGlyValArgAsnAsnLeuProIleSerPhelIleSerValSerTyrCysArgSerTyrThr

 Ndel (2189)
 2171 TATAAACTACTAAATTGCCATATGTACAATTCTGTAAGATAACAAAGTGTAAATATAATCAGGTAATAT
 599 TyrLysLeuLeuAsnCysHisMetTyrAsnSerCysLysIleThrLysCysTyrAsnGlnValIleT

 2241 ATAATCTATATAGGAGTATATAATTGAAAAGTAAAATATAATCATATAATAATGAAACGAATAT
 622 TyrAsnProIle...

 2311 CAGTAATAGACAGGAACGGCAGATTCTCTTAATGAAGTAAGTACTGCTAACATCTCCAAAATTAGAT

 2381 AAAATGATACAGCAAATACAGCTTCATTCAACGAATTACCTTTAATTTCAGACACACCTTAATTAC

 2451 AAACTAACTAAAGTCAGATGATGAGAAAAGTAAATATAAAATTAACTTATGGGTATAATATAATAAAAGATT

 2521 ATGATATTAATAATTACTAACGATGTTAAGACTTATTCCATCAACCCCTTCAAACCTTCTGGATA

 2591 TTATAAAATACCAAGTTAATGATATTAAAATAGATTGTTAAGAGATGTAATAATTATTGGAGGTAAAG

 2661 GATATAAAATTAGTCTATCTTCACATGGAAATGAATTACCTAATATAATAATTATGATAGGAATT

 2731 TAGGATTACAGCTGTTATATGTTAACACAATACAGCCAGATCTATGGTTATGGTAAAACACTGTAACGG

 2801 GAAGCAGCATTCTATGGTAACGGCCTATGTTAATAGCCAGATCATTTACTCTATAAACACATTTCACCA

 BamHI (2880)
 2871 CAAATAATAGGATCCTCTAGATATTAAATTATCTAACAAACAACAAAAAATTAAACGATGTATGGC

 2941 CAGAACTTCTACTAATAAAAGATAAAAGATAGTCTATCTTATCTAACAGATATGAAAGAAGATAATCA

 HindIII (3058)
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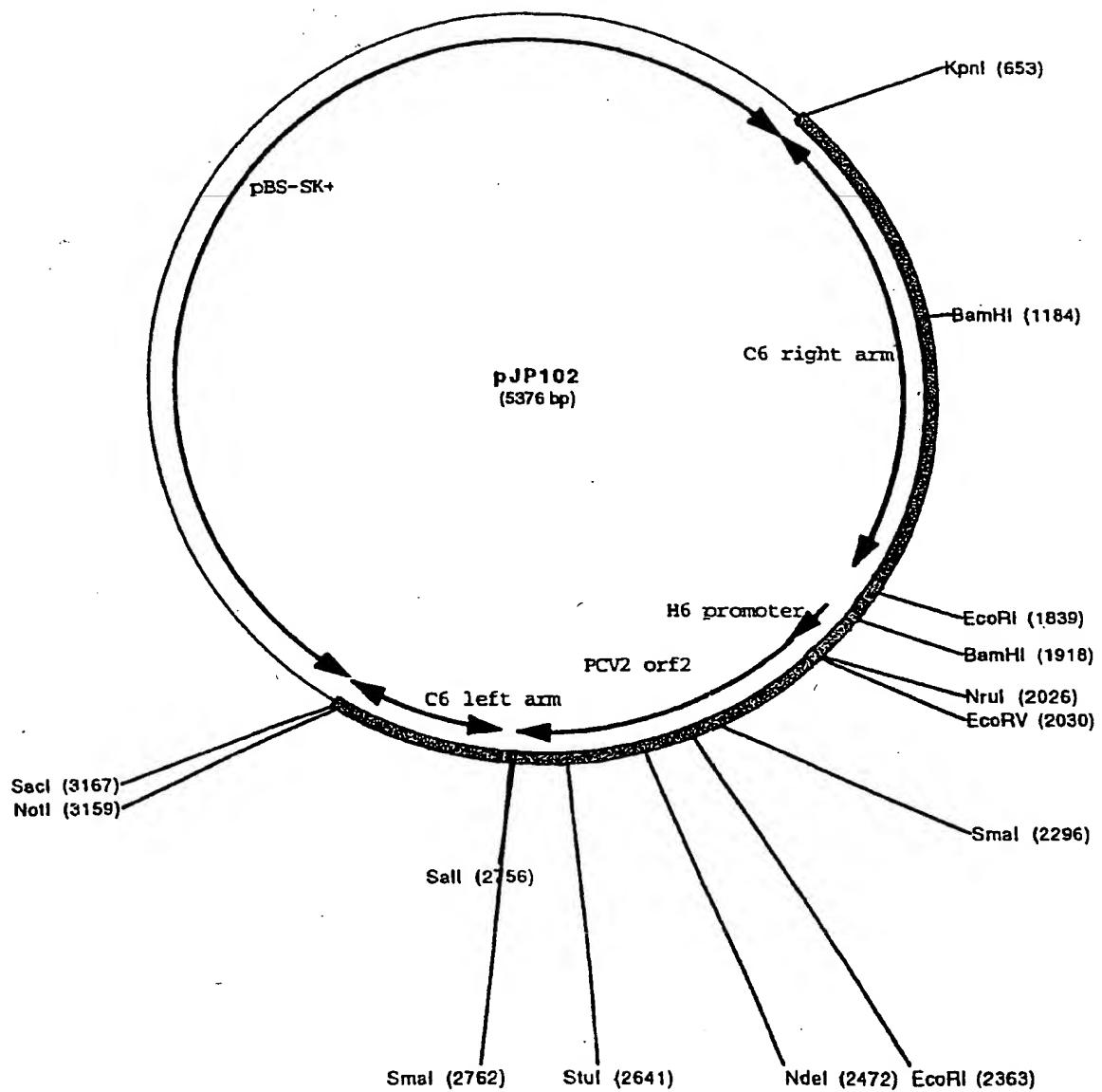
 3081 TTACTAGAAGATTAAAATCTAGACTTAGTATAACAAAACAGTTAAATGCCAATATCGATTCTATTTTC

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3221 CAATATGCCAATTATGTCTAATATTAACTTTAGAACTAAAACGTTCTACCAACTAAATAAGGATA
3291 CGTGATAGGCTGTTAAAAGCTGCAAAATAGTAAGGATGTAGAAGAAATACCTTGTTCTACCTTCGG
3361 AGGAAAGAACCTTAACTTAACTTAAGTTAACCTGAACTTGTATTATGAACACTATAAAAAATTATGGA
3431 AGATACAACTAAAAGAATGGATGTTGAATGTCGTAGTTAGAACATAACTATACGGCTAACCTTATATAAA
3501 GTGTACGGACAAAACGAATATATGATTACTTATATACTAGCTCTATAAGTAGGATTAATAATAATTATAG
3571 AAACTTAAAATATAATCTGGTGGGCTAGACGAATCTACAATACGTAATATAAATTATATAATTTCACA
3641 AAGAACAAAAAAATCAAGTTCTAACCTTATAGATAAACTATATTTCACCACTGA

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Fig 2



KpnI (1)

1 GGTACCTTCATAAAATACAAGTTGATTAACCTAAAGTTCTAAAGTTCTTCCCTCCGAAGGTATAGAA
 71 CAAAGTATTCTTCTACATCCTTACTATTATTGCAGCTTTACAGCTATCACGTATCTTATTTTTAG
 141 TATTGGTAGAACGTTTAGTTCTAAAGTTAAAATATTAGACATAATTGGCATATTGCTTATTCCTTGCAT
 211 AGTTGAGTCTGTAGATCGTTCAGTATATCACTGATTAATGTTACTGTGTTATGAAATATAGAATCG
 281 ATATTGGCATTTAACGTGTTGTTACTAAGTCTAGATTAAATCTTCTAGTAAATGCTATTTAATA
 351 TAAAAGCTTCCACGTTTGTTGATACATTTCTTCCATATTAGTAGCTACTAAATGATTATCTTCTT
 421 CATATCTTGTAGATAAGATAGACTATCTTATCTTATTAGTAGAAAATACTCTGGCCATACATCGTTA

BamHI (532)

491 AATTTTTTGTGTTGTTAGATATAATTTAAATATCTAGAGGATCTTATTGTTGGTAAAATGTTA
 561 TAGAGTAAAATGATCTGGCTATTAAACATAGGCCAGTTACCATAGAATGCTGCTCCCGTACAGTGT
 631 TACCATACCATAGATCTGCCTGTATTGTTGATACATATAACAGCTGTAATCTAAAAATTCTATCA
 701 TAATTATTAATATTAGGTAACTCATTCCATGTGAAAGATAGACTAATTATATCCTTACCTCCAAAT
 771 AATTATTTACATCTCTAAACAATCTATTAAATATCATTAACTGGTATTATAATATCCAGAAAGGTT
 841 TGAAGGGGGTGTGATGAAATAAGTCTATTAAACATCGTTAAGTAAATTATAATATCATGAATCTTATTATA
 911 TTATACCCATAAGTAAATTATAATTACTTCTCATCATCTGACTTAGTTAGTTGTAATAAGGTGTGT
 981 CTGAAAAAAATTAAAGGTAACTCGTTGAATGAAGCTGTTGCTGTATCTAATTGGAG
 1051 ATTTAGCAGTACTTACTTCATTAGAAGAATCTGCCAGTCTGCTTATTACTGATAATTCTGGTTCAT

EcoRI (1)

1121 TATTATATGATTATATTACTTTCAATTATATATACTCATTTGACTAGTTAATCAATAAAAGAAT
 1191 TCCPTGCAGCCCTGCGAGCTAATTAAATTAAAGCTACAAATACTTCTGTTTACCTTGTCTAAATAACTAATT

BamHI (1266)

1261 ATTAAGGATCCCCCAGCTTCTTATTCTATACCTAAAGTAAAATACAAAGGTTCTTGAGGGTT

EcoRV (1378)

NruI (1374)

1331 GTGTTAAATTGAAAGCGAGAAATAATCATAAAATTATTCTATTATCGCGATATCCGTTAAGTTGTATCGT
 1401 AATGACGTATCCAAGGAGGGCGTACCGCAGAAGAAGACACCGCCCCCGCAGCCATCTGGCCAGATCCTC
 1471 CGCCGCCGCCCTGGCTCGTCCACCCCCGCCACCGCTACCGTTGGAGAAGGAAAATGGCATCTCAACA
 241 ArgArgArgProTrpLeuValHisProArgHisArgTyrArgTrpArgArgLysAsnGlyIlePheAsnT
 1541 CCCGCCTCTCCCGCACCTTCGGATATACTGTCAAGCGTACCCACAGTCACAAAGCCCTCTGGCGGTGGA
 471 ArgLeuSerArgThrPheGlyTyrThrValLysArgThrThrValThrThrProSerTrpAlaValAs

SmaI (1644)

1611 CATGATGAGATTTAAATTGACGACTTTGTTCCCCCGGGAGGGGGGACCAACAAAATCTCTATACCCCTT
 701 pMetMetArgPheLysIleAspAspPheValProProGlyGlyThrAsnLysIleSerIleProPhe

EcoRI (1711)

1681. GAATACTACAGAATAAGAAAGGTTAAGGTTGAATCTGGCCCTGCTCCCCCATCACCCAGGGTGTAGGG
 94> Glu Tyr Tyr Arg Ile Arg Lys Val Lys Val Glu Phe Trp Pro Cys Ser Pro Ile Thr Glu Gly Asp Arg G

NdeI

1751. GAGTGGGCCACTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCACAGCCCTAACCTATGACCC
 117> Ile Val Glu Ser Thr Ala Val Ile Leu Asp Asp Asn Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr Asp Pro

1821. ATATGTAACACTACTCCCTCCGCCATACAATCCCCAACCTTCTCCTACCACCTCCCGTTACTTCACACCC
 140> Pro Tyr Val Asn Tyr Ser Ser Arg His Thr Ile Pro Glu Pro Phe Ser Tyr His Ser Arg Tyr Phe Thr Pro

1891. AAACCTGTTCTTGACTCCACTATTGATTACTTCCAACCAAATAACAAAAGGAATCAGCTTGGCTGAGAC
 164> Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Glu Pro Asn Asn Lys Arg Asn Glu Leu Trp Leu Arg L

SstI (1989)

1961. TACAAACCTCTGGAAATGTGGACCACGTAGGCCCTCGGCCCTCGCTTCAACAGTAAATACGACCAGGA
 187> Glu Ile Thr Ser Glu Asn Val Asp His Val Glu Leu Glu Ala Ala Phe Glu Asn Ser Lys Tyr Asp Glu As

2031. CTACAATATCCGTGTAACCATGTATGTACAATTCAAGAGAATTAACTTAAAGACCCCCACTTAAACCC
 210> Pro Tyr Asn Ile Arg Val Thr Met Tyr Val Glu Phe Arg Glu Phe Asn Leu Lys Asp Pro Pro Leu Lys Pro

SmaI (2110)

SalI (2104)

2101. TAAGTCGACCCCGGGTTTTATAGCTAATTAGTCATTTTCTCGTAAGTAAGTATTTTATTAATACTTT

2171. TTATTGTACTTATGTTAAATATAACTGATGATAACAAATCATTATGTATTATTTATAACTGTAATTTC

2241. TTTAGCGTAGTTAGATGTCAAATCTCTCTCAAATACATCGGCTATCTTGTAGTGAGATTGTGATCTATG

2311. CAGTTGAAACTTATGAACCGGTGATGATTAAATGTGAACCGTCAAATTGCACTTATGAGCGT

2381. ATCTATTATCTACTATCATCTTGTAGTTATTAAATCATCTACTTTAGAATTGATAGGAATATGAA

SacI (2515)

NotI (2507)

2451. TACCTTTGTAGTAATATCTATACTATCTACACCTAACTCATTAAGACTTTGATAGGCCGCCGAGCTC

Fig. 3B

Fig. 4

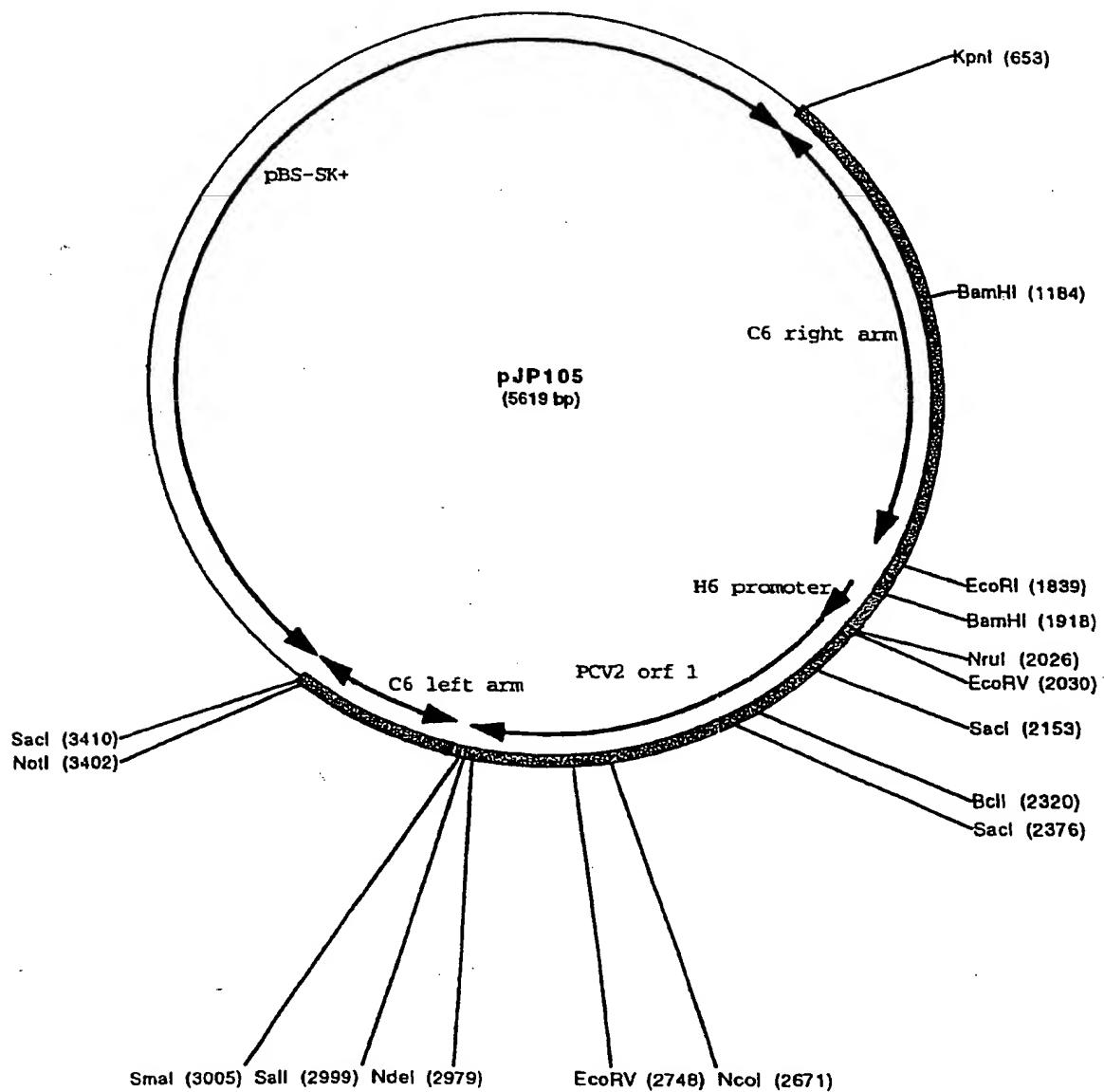


Fig 6A

KpnI (1)

1 GGTACCTTCATAAAATACAAGTTGATTAACTTAAGTTGTTCTAAAGTTCTTCCCGAAGGTATAGAA
 71 CAAAGTATTCTTCTACATCCTTACTATTTATTCAGCTTTAACAGCCTATCACGTATCCTATTTAG
 141 TATTGGTAGAACGTTTACTGTTCTAAAGTTAAAATTTAGACATAATTGGCATATTGCTTATTCCTTGCAT
 211 AGTTGAGCTGTAGATCGTTCACTGATATCACTGATTAATGTTACTGTATGATGAAATATAGAAATCG
 281 ATATTGGCATTAACTGTTGTTACTAAGTCTAGATTTAAATCTTCTAGTAATATGCTATTAAATA
 351 TAAAAGCTTCCACGTTTGTATACATTTCCATATTAGTAGCTACTACTAAATGATTATCCTT
 421 CATATCTTGTAGATAAGATAGACTATCTTATCTTATTAGTAGAAAATACTTCTGGCCATACATCGTTA

BamHI (532)

491 AATTTTTTTGTGTGTGTAGATATAATTTAAATATCTAGAGGATCTTATTGTGGTAAAATGTTA
 561 TAGAGTAAAATGATCTGGCTATTAACATAGGCCAGTTACCATAGAAATGCTGCTCCGTTACAGTGT
 631 TACCATAACCATAGATCTGCCGTATGTGATAACATATAACAGCTGTAATTCTAAAAAATCCTATCA
 701 TAATTATAATATTAGGTAATTCAATTCCATGTGAAAGATAGACTAATTTCATACCTTACCTCCAAAT
 771 AATTATTACATCTTAAACAATCTATTAAATATCAATTACTGGTATTATAATATCCAGAAAGGT
 841 TGAAGGGTTGATGGAATAAGTCTATTAAACATCGTTAAGTAAATTATAATATCATGAATCTTATTATA
 911 TTATACCCATAAGTTAAATTATTTACTTCTCATCATCTGACTTAGTTAGTTGTAATAAGGTGT
 981 CTGAAAAAATTAAAAGGTAACTCGTGAATGAAGCTGTTGCTGTATCTAAATTGGAG
 1051 ATTTAGCAGTACTTACTTCATTAGAAGAAGAATCTGCCAGTTCTGTCTATTACTGATAATTCTGTTCAT
 1121 TATTATATGATTATATTACTTTCAATTATATATCAATTGACTAGTTAATCAATAAAAAGAAT
 1191 TTGACTTGGGTTAAGTGGGGGCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTACACGG
 233 ProLysLeuProProAspLysLeuAsnPheGluArgPheGlyValTyrMetThrValArg

SstI (1306)

1261 ATATTGTACTCCTGGTCTGTAATTACTGTTTGCACGCCAGCGCCGAGGCCCTACGTGGTCACATTCCAG
 212 LeAsnTyrAspGlyAspTyrLysSerAsnGluPheAlaAlaGlyLeuGlyValHisAspValAsnGlySe
 1331 AGGTTTGTAGTCAGCCAAAGCTGATTCTTGTATTGGTTGGAAGTAAATCAATAGTGGAGTCAG
 189 ThrGlyLeuArgLeuTrpLeuGlyAsnArgLysAsnAsnProGlyPheTyrAspIleThrSerAspLeu
 1401 AACAGGTTGGGTGTGAAGTAACGGGAGTGGTAGGGAGAAGGGTTGGGGATTGTATGGGGGGAGGAGTAG
 166 ValProLysProThrPheTyrArgSerHisTyrSerPheProGlyProGlyProIleThrHisArgSerSerTyrA

NdeI (1475)

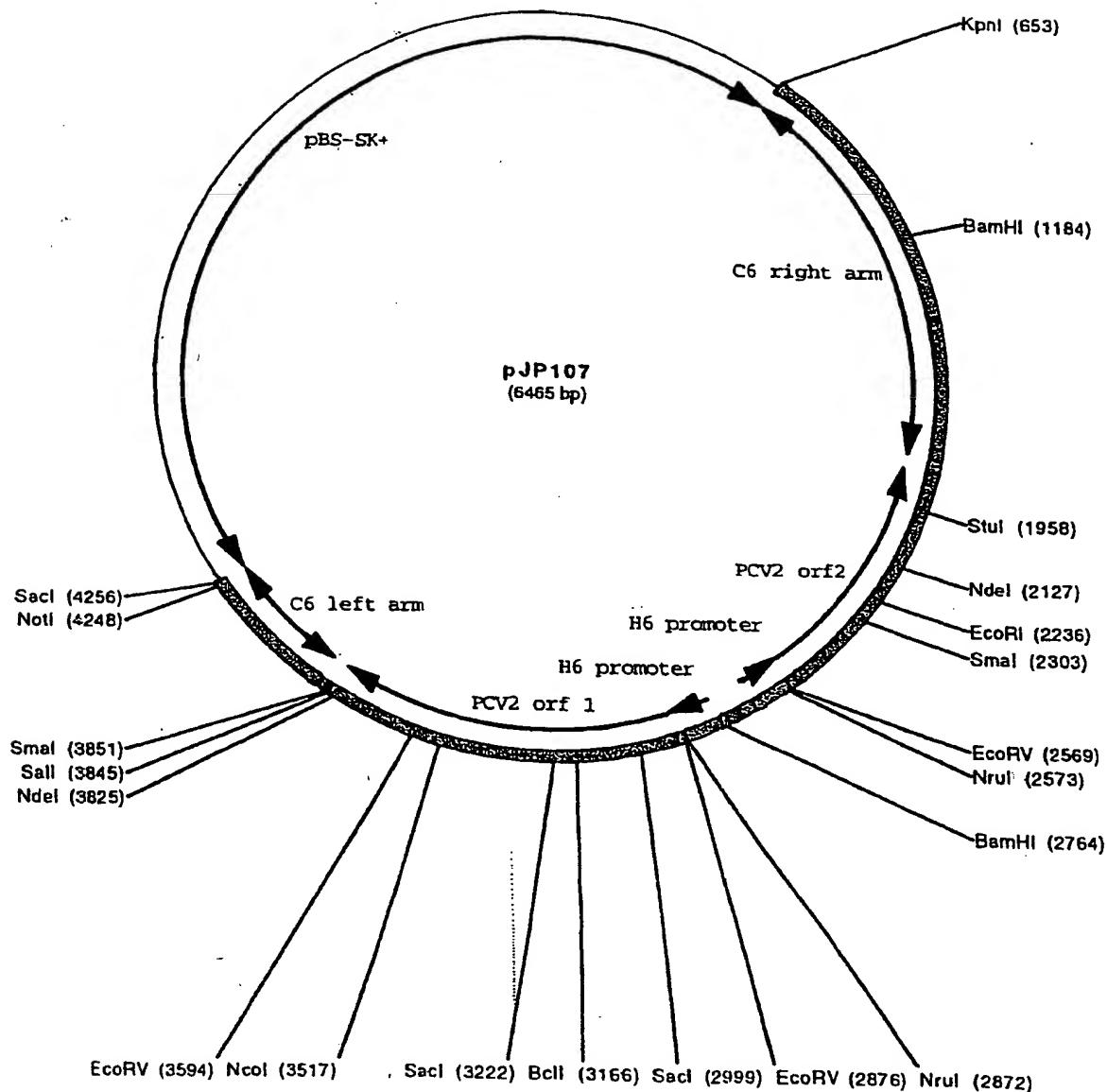
1471 TTTCACATATGGGTCTAGGGTAGGGCTGTGGCCCTTGTACAAAGTTATCATCTAGAATAACAGCAGTGG
 142 AsnValTyrProAspTyrThrLeuAlaThrAlaLysThrValPheAsnAspAspLeuIleValAlaThrSe

EcoRI (1584)

1541 AGCCCCACTCCCCATACCCCTGGGTGATGGGGAGCCAGGGCCAGAAATCAGCTAACCTTCTTATCT
 119 GlyValGlyArgAspGlyGlyThrIleProSerCysProTrpPheGlyValLysValLysArgIleArg

SmaI (1651)

1611 GTAGTATTCAAAGGGTATAGAGATTGGTAGGTCCCCCTGCCGGGGAAACAAAGTCGTCAATTAAAT
 96 TyrTyrGlyPheProIleSerIleLysAsnThrGlyGlyProProValPheAspIleLysPheA

9/11
Fig 5

10/11

1681 CTCATCATGTCCACCGCCCAAGGAGGGCGTTGTGACTGTGGTACGCTTGACAGTATATCCGAAGGTGGGG
 724 r gMet Met Asp Val I A l r p Ser Pro Th r Th r Val Th r Th r Arg Lys Val Th r Ty r Gly Phe Th r Arg Se

1751 AGAGGCAGGGTGTGAAAGATGCCATTTCCTCTCCAAACGGTAGCGGGTGGCGGGGGTGGACGAGCCAGGG
 494 r Leu Arg Th r Asn Phe l l e G l y Asn Lys Arg Arg T r p Arg Ty r Arg His Arg Pro His Val Leu T r p Pro

1821 GCGGGCGGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTCTGCGGTAAACGCCCTCCCTGGGA
 264 r Arg Arg Arg Leu l l e G l n G l y Leu His Ser Arg Pro Arg His Arg Arg Arg Arg Ty r Arg Arg Arg Pro T

NruI (1921)
 EcoRV (1917)

1891 TACGTCAATTACGATAACAAACTTAACGGATATCGCGATAATGAAATAATTATGATTATTCTCGCTTCA
 244 r Th r Met

1961 ATTTAACACAACCCCTCAAGAACCTTGATTTTACTTTTAAGTATAGAATAAGAACGCTGGGG

2031 ATCAATTCTGCAAGCCCTGCAGCTAAATTAAAGCTACAAATAGTTCTGTTTCACTTGTCTAAATAAC

BamHI (2112)

2101 TAATTAATTAAGGATCCCCAGCTCTTTATTCTATACTTAAAAAGTAAAAATAACAAAGGTTCTG

EcoRV (2224)
 NruI (2220)

2171 AGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAAATTATTCTATTGATATCCGATATCCGTTAAGTTG

2241 TATCGTAATGCCAGCAAGAAGAAATGGAAGAACGGGACCCCAACCACATAAAAGGTGGGTGTTACGCTG
 1 r Met Pro Ser Lys Lys Asn G l y Arg Ser G l y Pro G l n Pro His Arg T r p Val I Phe Th r Leu

SacI (2347)

2311 AATAATCCTCCGAAGACGAGCGCAAGAAAATACGGGAGCTCCAATCTCCCTATTGATTATTATTG
 22 r Asn Asn Pro Ser G l u Asp G l u Arg Lys Lys l l e Arg G l u Leu Pro l l e Ser Leu Phe Asp Ty r Phe l l e V

2381 TTGGCGAGGGAGGGTAATGAGGAAGGAGCAACACCTCACCTCCAGGGTTCCCTAATTGTAAGAACCA
 45 r al G l y G l u G l y Asn G l u G l y Arg Th r Pro His Leu G l n G l y Phe Al a Asn Phe Val I Lys G l y

BclI (2514)

2451 AACTTTAAATAAACGAAAGTGGTATTGGGTGCCGCTGCCACATCGAGAAAGCAAAGGAACTGATCAG
 68 r n Th r Phe Asn Lys Val I Lys T r p Ty r Leu G l y Al a Arg Cys His l l e G l u Lys Al a Lys G l y Th r Asp G l n

SacI (2570)

2521 CAGAATAAAAGAATATTGCACTAAAGAAGGCAACTTACTTATGAAATGTGGAGCTCTGATCTCAAGGAC
 92 r G l n Asn Lys G l u Tyr Cys Ser Lys G l u G l y Asn Leu Leu l l e G l u Cys G l y Al a Pro Arg Ser G l n G l y G

2591 AACGGAGTGACCTGCTACTGCTGTGAGTACCTTGTTGGAGAGCGGGAGTCTGGTGACCGTTGCAGAGCA
 115 r l n Arg Ser Asp Leu Ser Th r Al a Val Ser Th r Leu Leu G l u Ser G l y Ser Leu Val Th r Val I A l a G l u G l

2661 GCACCCCTGTAACGTTGTCAGAAATTCGGCGGGCTGGCTGAACCTTGTAAAGTGACCGGGAAAATGCAG
 138 r n His Pro Val Th r Phe Val I A rg Asn Phe Arg G l y Leu Al a G l u Leu Leu Lys Val I Ser G l y Lys Met G l n

2731 AAGCGTGATTGGAAGACCAATGTACACGTCATTGTTGGGCCACCTGGGTGTGGTAAAGCAAATGGCTG
 162 r Lys Arg Asp T r p Lys Th r Asn Val I His Val I l l e Val I G l y Pro P r o G l y Cys G l y Lys Ser Lys T r p Al a A

NcoI (2865)

2801 CTAATTTGCAGACCCGGAAACACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGG
 185 r l a Asn Phe Al a Asp Pro G l u Th r Th r Ty r T r p Lys Pro Pro Arg Asn Lys T r p T r p Asp G l y Ty r His G l

2871 TGAAGAAAGTGGTGTATTGATGACTTTATGGCTGGCTGCCGTGGGATGATCTACTGAGACTGTGTGAT
 208 r y G l u G l u Val I Val I Val I l l e Asp Asp Phe Ty r G l y T r p Leu Pro T r p Asp Asp Leu Leu Arg Leu Cys Asp

EcoRV (2942)

2941 CGATATCCATTGACTGTAGAGACTAAAGGTGGAACGTGACCTTTGGCCCGCAGTATTCTGATTACCA
 232 r Arg Ty r Pro Leu Th r Val I G l u Th r Lys G l y Th r Val Pro Phe Leu Al a Arg Ser l l e Leu l l e Th r S

3011 GCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTGCCCCAGCTGTAGAAGCTCTATCGGAGGAT
255► er Asn Glu Thr Pro Leu Glu Trp Tyr Ser Ser Thr Ala Val Pro Ala Val Glu Ala Leu Tyr Arg Arg II

3081 TACTTCCTGGTATTTGGAAAGAATGGTACAGAACAAATCCACGGAGGAACGGGGCCAGTTCTCACCCCTT
278► e Thr Ser Leu Val Phe Trp Lys Asn Ala Thr Glu Glu Ser Thr Glu Glu Glu Glu Glu Phe Val Thr Leu

SmaI (3199)

NdeI (3173) Sall (3193)

3151 TCCCCCCCCATGCCCTGAATTTCCATATGAAATAAAATTACTGAGTCGACCCCCGGTTTATAGCTAATTA
302► Ser Pro Pro Cys Pro Glu Phe Pro Tyr Glu Ile Asn Tyr

3221 GTCATTTTCGTAAGTAAGTATTTTATTTAACTTTTATTTGACTTATGTTAAATATAACTGATGA

3291 TAACAAAATCCATTATGTATTATTTATAACTGTAATTCTTTAGCGTAGTTAGATGTCCAATCTCTCA

3361 AATACATCGGCTATCTTTAGTGAGATTTGATCTATGCAGTTGAAACTTATGAACCGGTATGATTAA

3431 AATGTGAACCGTCCAAATTGCACTTATATGAGCGTATCTATTATCTACTATCATCATCTTGAGTT

3501 ATTAATATCATCTACTTTAGAATTGATAGGAAATATGAATACCTTGAGTAATATCTATACTATCTACA

SacI (3604)

NolI (3596)

3571 CCTAACTCATTAAGACTTTGATAGGCGGCCGCGAGCTC

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Bublot, Michel
Perez, Jennifer M.
Charreyre, Catherine E.

(ii) TITLE OF INVENTION: Porcine Circovirus 2 Recombinant
Poxvirus

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: ~~Virogenetics Inc~~ Frommer Lawrence & Haug LLP
(B) STREET: 465 Jordan Road 745 Fifth Avenue
(C) CITY: ~~Troy~~ NY
(D) STATE: NY NY
(E) COUNTRY: USA
(F) ZIP: 12180 10151

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: ~~Hewe~~ Timothy P. Kowalski, Thomas J.
(B) REGISTRATION NUMBER: 39,228 32,147
(C) REFERENCE/DOCKET NUMBER: TH015 454313-2511.1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (570) 586-1022 212-588-0800
(B) TELEFAX: (570) 895-2702 212-588-0500

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3701 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTCTAT CAAAAGTCTT AATGAGTTAG GTGTAGATAG TATAGATATT ACTACAAAGG
60

TATTCAATT TCCTATCAAT TCTAAAGTAG ATGATATTAA TAACTCAAAG ATGATGATAG
120

TAGATAATAG ATACGCTCAT ATAATGACTG CAAATTGGA CGGTTCACAT TTTAATCATC
180

ACCGGTTCAT AAGTTCAAC TGCATAGATC AAAATCTCAC TAAAAAGATA GCCGATGTAT
240

TTGAGAGAGA TTGGACATCT AACTACGCTA AAGAAATTAC AGTTATAAT AATACATAAT
300

GGATTTGTT ATCATCAGTT ATATTAACA TAAGTACAAT AAAAGTATT AAATAAAAAT
360

ACTTACTTAC GAAAAATGT CATTATTACA AAAACTATAT TTTACAGAAC AATCTATAGT
420

AGAGTCCTT AAGAGTTATA ATTTAAAAGA TAACCATAAT GTAATATTAA CCACATCAGA
480

TGATGATACT GTTGTAGTAA TAAATGAAGA TAATGACTG TTATCTACAA GATTATTATC
540

ATTTGATAAA ATTCTGTTT TAAACTCCTT TAATAACGGT TTATCAAAT ACGAAACTAT
600

TAGTGATACA ATATTAGATA TAGATACTCA TAATTATTAT ATACCTAGTT CTTCTTCTTT

660

GTTAGATATT CTA##### GAGCGTGTGA TTTAGAATTA GAAGATCTAA ATTATGCGTT
720

AATAGGAGAC AATAGTAACT TATATTATAA AGATATGACT TACATGAATA ATTGGTTATT
780

TACTAAAGGA TTATTAGATT ACAAGTTGT ATTATTGCGC GATGTAGATA AATGTTACAA
840

ACAGTATAAT AAAAAGAATA CTATAATAGA TATAATACAT CGCGATAACA GACAGTATAA
900

CATATGGGTT AAAAATGTTA TAGAATACTG TTCTCCTGGC TATATATTAT GGTTACATGA
960

TCTAAAAGCC GCTGCTGAAG ATGATTGGTT AAGATACGAT AACCGTATAA ACGAATTATC
1020

TGCGGATAAA TTATACACTT TCGAGTTCAT AGTTATATTA GAAAATAATA TAAAACATTT
1080

ACGAGTAGGT ACAATAATTG TACATCCAA CAAGATAATA GCTAATGGTA CATCTAATAA
1140

TATACTTACT GATTTCTAT CTTACGTAGA AGAACTAATA TATCATCATA ATTCACTAT
1200

AATATTGGCC GGATATTTTT TAGAATTCTT TGAGACCACT ATTTTATCAG AATTTATTC
1260

TTCATCTTCT GAATGGTAA TGAATAGTAA CTGTTAGTA CACCTGAAAA CAGGGTATGA
1320

AGCTATACTC TTTGATGCTA GTTTATTTT CCAACTCTCT ACTAAAAGCA ATTATGTAAA
1380

ATATTGGACA AAGAAAACCTT TGCAGTATAA GAACTTTTT AAAGACGGTA AACAGTTAGC
1440

AAAATATATA ATTAAGAAAG ATAGTCAGGT GATAGATAGA GTATGTTATT TACACGCAGC
1500

TGTATATAAT CACGTAACIT ACTTAATGGA TACGTTAAA ATTCTGGTT TTGATTTAA
1560

ATTCTCCGGA ATGATAGATA TACTACTGTT TGGAATATTG CATAAGGATA ATGAGAATAT
1620

ATTTTATCCG AAACGTGTTT CTGTAACTAA TATAATATCA GAATCTATCT ATGCAGATTT
1680

TTACTTTATA TCAGATGTTA ATAAATTCAG TAAAAAGATA GAATATAAAA CTATGTTCC
1740

TATACTCGCA GAAAACTACT ATCCAAAAGG AAGGCCCTAT TTTACACATA CATCTAACGA
1800

AGATCTTCTG TCTATCTGTT TATGCGAAGT AACAGTTGT AAAGATATAA AAAATCCATT
1860

ATTATATTCT AAAAAGGATA TATCAGCAAA ACGATTACATA GGTTTATTAA CATCTGTCGA
1920

TATAAATACG GCTGTTGAGT TAAGAGGATA TAAAATAAGA GTAATAGGAT GTTACAATG
1980

GCCTGAAAAG ATAAAAATAT TTAATTCTAA TCCTACATAC ATTAGATTAT TACTAACAGA
2040

AAGACGTTA GATATTCTAC ATTCTATCT GCTTAAATTT AATATAACAG AGGATATAGC
2100

TACCAGAGAT GGAGTCAGAA ATAATTACCC TATAATTCTT TTTATCGTCA GTTATTGTAG
2160

ATCGTACT TATAAATTAC TAAATTGCCA TATGTACAAT TCGTGTAAGA TAACAAAGTG
2220

TAAATATAAT CAGGTAATAT ATAATCCTAT ATAGGAGTAT ATATAATTGA AAAAGTAAAA
2280

TATAAATCAT ATAATAATGA AACGAAATAT CAGTAATAGA CAGGAACTGG CAGATTCTC
2340

TTCTAATGAA GTAAGTACTG CAAATCTCC AAAATTAGAT AAAATGATA CAGCAAATAC
2400

AGCTTCATTC AACGAATTAC CTTTTAATT TTTCAGACAC ACCTTATTAC AACTAACTA
2460

AGTCAGATGA TGAGAAAGTA AATATAAATT TAACTTATGG GTATAATATA ATAAAGATTG
2520

ATGATATTAA TAATTTACTT AACGATGTTA ATAGACTTAT TCCATCAACC CCTTCAAACC
2580

TTTCTGGATA TTATAAAATA CCAGTTAATG ATATTAAAAT AGATTGTTA AGAGATGTAA
2640

ATAATTATTT GGAGGTAAG GATATAAAAT TAGTCTATCT FTCACATGGA AATGAATTAC
2700

CTAATATTAA TAATTATGAT AGGAATTTTT TAGGATTAC AGCTGTTATA TGTATCAACA
2760

ATACAGGCAG ATCTATGGTT ATGGTAAAC ACTGTAACGG GAAGCAGCAT TCTATGGTAA
2820

CTGGCCTATG TTTAATAGCC AGATCATTTC ACTCTATAAA CATTACCA CAAATAATAG
2880

GATCCTCTAG ATATTTAATA TTATATCTAA CAACAACAAA AAAATTTAAC GATGTATGGC
2940

CAGAAGTATT TTCTACTAAT AAAGATAAAG ATAGTCTATC TTATCTACAA GATATGAAAG
3000

AAGATAATCA TTTAGTAGTA GCTACTAATA TGGAAAGAAA TGTATACAAA AACGTGGAAG
3060

CTTTTATATT AAATAGCATA TTACTAGAAG ATTTAAAATC TAGACTTAGT ATAACAAAAC
3120

AGTTAAATGC CAATATCGAT TCTATATTC ATCATAACAG TAGTACATTA ATCAGTGATA
3180

TACTGAAACG ATCTACAGAC TCAACTATGC AAGGAATAAG CAATATGCCA ATTATGTCTA

3240

ATATTTAAC TTTAGAACTA AAACGTTCTA CCAACTAA AAATAGGATA CGTGATAGGC
3300

TGTTAAAAGC TGCAATAAAT AGTAAGGATG TAGAAGAAAT ACTTTGTTCT ATACCTTCGG
3360

AGGAAAGAAC TTTAGAACAA CTTAAGTTA ATCAAACCTG TATTTATGAA CACTATAAAA
3420

AAATTATGGA AGATACAAGT AAAAGAATGG ATGTTGAATG TCGTAGTTA GAACATAACT
3480

ATACGGCTAA CTTATATAAA GTGTACGGAC AAAACGAATA TATGATTACT TATATACTAG
3540

CTCTCATAAG TAGGATTAAT AATATTATAG AAACTTAAA ATATAATCTG GTGGGGCTAG
3600

ACGAATCTAC AATACGTAAT ATAAATTATA TAATTCACA AAGAACAAAA AAAAATCAAG
3660

TTTCTAATAC CTTATAGATA AACTATATTT TTTACCACTG A
3701

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCATCGAGC TCGCGGCCGC CTATCAAAG TCTTAATGAG TT
42

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCCCTCG AGCTGCAGCC CGGGTTTTTA TAGCTAATTA GTCATTTTT CGTAAGTAAG
60

TATTTTTATT TAA

73

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 72 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCGGGCTGC AGCTCGAGGA ATTCTTTTA TTGATTAAGT AGTCAAATGA GTATATATAA
60

TTGAAAAAGT AA

72

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATGATGGTA CCTTCATAAA TACAAGTTG ATTAAACTTA AGTTG
45

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATCATCATG ATATCCGTTA AGTTTGTATC GTAATGACGT ATCCAAGGAG GCG
53

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TACTACTACG TCGACTTAGG GTTTAAGTGG GGGGTC
36

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2520 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGTACCTTCA TAAATACAAG TTTGATTAAA CTTAAGTTGT TCTAAAGTTC TTTCCCTCCGA
60

AGGTATAGAA CAAAGTATTT CTTCTACATC CTTACTATTT ATTGCAGCTT TTAACAGCCT
120

ATCACGTATC CTATTTTAG TATTGGTAGA ACGTTTAGT TCTAAAGTTA AAATATTAGA
180

CATAATTGGC ATATTGCTTA TTCCCTGCAT AGTTGAGTCT GTAGATCGTT TCAGTATATC
240

ACTGATTAAT GTACTACTGT TATGATGAAA TATAGAATCG ATATTGGCAT TTAACTGTTT
300

TGTTTACTA AGTCTAGATT TTAAATCTTC TAGTAATATG CTATTTAATA TAAAAGCTTC
360

CACGTTTTG TATACATTTC TTCCATATT AGTAGCTACT ACTAAATGAT TATCTTCTTT
420

CATATCTTGT AGATAAGATA GACTATCTT ATCTTTATTA GTAGAAAATA CTTCTGGCCA
480

TACATCGTTA AATTTTTTG TTGTTGTTAG ATATAATATT AAATATCTAG AGGATCCTAT
540

TATTTGTGGT AAAATGTTA TAGAGTAAAA TGATCTGGCT ATTAAACATA GGCCAGTTAC
600

CATAGAATGC TGCTTCCCGT TACAGTGTTC TACCATAACC ATAGATCTGC CTGTATTGTT
660

GATACATATA ACAGCTGTAA ATCCTAAAAA ATTCCTATCA TAATTATTA TATTAGGTAA
720

TTCATTTCCA TGTGAAAGAT AGACTAATT TATATCCTTT ACCTCCAAAT AATTATTTAC
780

ATCTCTTAAA CAATCTATTT TAATATCATT AACTGGTATT TTATAATATC CAGAAAGGTT
840

TGAAGGGGTT GATGGAATAA GTCTATTAAC ATCGTTAAGT AAATTATTAA TATCATGAAT
900

CTTTATTATA TTATACCCAT AAGTTAAATT TATATTACT TTCTCATCAT CTGACTTAGT
960

TAGTTTGTAA TAAGGTGTGT CTGAAAAAAT TAAAAGGTAA TTCGTTGAAT GAAGCTGTAT
1020

TTGCTGTATC ATTTTTATCT AATTTGGAG ATTTAGCAGT ACTTACTTCA TTAGAAGAAG
1080

AATCTGCCAG TTCCTGTCTA TTACTGATAT TTCGTTCAT TATTATATGA TTTATATTTT
1140

ACTTTTCAA TTATATATAC TCATTTGACT AGTTAATCAA TAAAAAGAAT TCCTGCAGCC
1200

CTGCAGCTAA TTAATTAAGC TACAAATAGT TTCGTTTCA CCTTGTCTAA TAACTAATTA
1260

ATTAAGGATC CCCCAGCTTC TTTATTCTAT ACTTAAAAAG TGAAAATAAA TACAAAGGTT
1320

CTTGAGGGTT GTGTTAAATT GAAAGCGAGA AATAATCATA AATTATTTCA TTATCGCGAT
1380

ATCCGTTAAG TTTGTATCGT AATGACGTAT CCAAGGAGGC GTTACCGCAG AAGAAGACAC
1440

CGCCCCCGCA GCCATCTTGG CCAGATCCTC CGCCGCCGCC CCTGGCTCGT CCACCCCCGC
1500

CACCGCTACC GTTGGAGAAG GAAAAATGGC ATCTCAACA CCCGCCTCTC CCGCACCTTC
1560

GGATATACTG TCAAGCGTAC CACAGTCACA ACGCCCTCCT GGGCGGTGGA CATGATGAGA
1620

TTTAAAATTG ACGACTTGT TCCCCCGGGA GGGGGGACCA ACAAAATCTC TATACCCCTT
1680

GAATACTACA GAATAAGAAA GGTTAAGGTT GAATTCTGGC CCTGCTCCCC CATCACCCAG
1740

GGTGATAGGG GAGTGGGCTC CACTGCTGTT ATTCTAGATG ATAACCTTGT AACAAAGGCC
1800

ACAGCCCTAA CCTATGACCC ATATGTAAAC TACTCCTCCC GCCATACAAT CCCCCAACCC
1860

TTCTCCTACC ACTCCGTTA CTTCACACCC AAACCTGTT TTGACTCCAC TATTGATTAC
1920

TTCCAACCAA ATAACAAAAG GAATCAGCTT TGGCTGAGAC TACAAACCTC TGGAAATGTG
1980

GACCACGTAG GCCTCGCGC TGCCTCGAA AACAGTAAAT ACGACCAGGA CTACAATATC
2040

CGTGTAAACCA TGTATGTACA ATTCAAGAGAA TTTAATCTTA AAGACCCCCC ACTTAAACCC
2100

TAAGTCGACC CCGGGTTTTT ATAGCTAATT AGTCATTTT TCGTAAGTAA GTATTTTAT
2160

TTAATACTTT TTATTGTACT TATGTTAAAT ATAACGTGATG ATAACAAAAT CCATTATGTA
2220

TTATTATCAA CTGTAATTTC TTTAGCGTAG TTAGATGTCC AATCTCTCTC AAATACATCG
2280

GCTATCTTT TAGTGAGATT TTGATCTATG CAGTTGAAAC TTATGAACGC GTGATGATTA
2340

AAATGTGAAC CGTCCAAATT TGCAGTCATT ATATGAGCGT ATCTATTATC TACTATCATC
2400

ATCTTGAGT TATTAATATC ATCTACTTTA GAATTGATAG GAAATATGAA TACCTTTGTA
2460

GTAATATCTA TACTATCTAC ACCTAACTCA TTAAGACTTT TGATAGGCAG CCGCGAGCTC

2520

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 57 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATCATCATT CGCGATATCC GTTAAGTTTG TATCGTAATG CCCAGCAAGA AGAATGG
57

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACTACTACG TCGACTCAGT AATTATTTTC ATATGG
36

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3609 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGTACCTTC AAAATACAAG TTTGATTAAA CTTAAGTTGT TCTAAAGTTC TTTCCCTCCGA

60

AGGTATAGAA CAAAGTATTT CTTCTACATC CTTACTATTT ATTGCAGCTT TTAACAGCCT
120ATCACGTATC CTATTTTAG TATTGGTAGA ACGTTTAGT TCTAAAGTTA AAATATTAGA
180CATAATTGGC ATATTGCTTA TTCCCTGCAT AGTTGAGTCT GTAGATCGTT TCAGTATATC
240ACTGATTAAT GTACTACTGT TATGATGAAA TATAGAATCG ATATTGGCAT TTAACTGTTT
300TGTTATACTA AGTCTAGATT TAAATCTTC TAGTAATATG CTATTTAATA TAAAAGCTTC
360CACGTTTTG TATACATTTC TTTCCATATT AGTAGCTACT ACTAAATGAT TATCTCTTT
420CATATCTTGT AGATAAGATA GACTATCTT ATCTTTATTA GTAGAAAATA CTTCTGGCCA
480TACATCGTTA AATTTTTTG TTGTTGTTAG ATATAATATT AAATATCTAG AGGATCCTAT
540TATTTGTGGT AAAATGTTA TAGAGTAAAA TGATCTGGCT ATTAAACATA GGCCAGTTAC
600CATAGAATGC TGCTTCCCGT TACAGTGTTC TACCATAACC ATAGATCTGC CTGTATTGTT
660GATACATATA ACAGCTGTAA ATCCTAAAAA ATTCCTATCA TAATTATTA TATTAGGTAA
720TTCATTTCCA TGTGAAAGAT AGACTAATTT TATATCCTTT ACCTCCAAAT AATTATTTAC
780ATCTCTTAAA CAATCTATTT TAATATCATT AACTGGTATT TTATAATATC CAGAAAGGTT
840

TGAAGGGTT GATGGAATAA GTCTATTAAC ATCGTTAAGT AAATTATTAA TATCATGAAT
900

CTTTATTATA TTATACCCAT AAGTTAAATT TATATTACT TTCTCATCAT CTGACTTAGT
960

TAGTTTGTAA TAAGGTGTGT CTGAAAAAAT TAAAAGGTAA TTCTGTTGAAT GAAGCTGTAT
1020

TTGCTGTATC ATTTTTATCT AATTTGGAG ATTTAGCAGT ACTTACTTCA TTAGAAGAAG
1080

AATCTGCCAG TTCCTGTCTA TTACTGATAT TTCTGTTCAT TATTATATGA TTTATATTTT
1140

ACTTTTCAA TTATATATAC TCATTTGACT AGTTAATCAA TAAAAGAAT TTCTGACTTAG
1200

GGTTTAAGTG GGGGTCTTT AAGATTAAAT TCTCTGAATT GTACATACAT GGTTACACGG
1260

ATATTGAGT CCTGGTCGTA TTTACTGTTT TCGAACGCAG CGCCGAGGCC TACGTGGTCC
1320

ACATTTCCAG AGGTTTGTAG TCTCAGCCAA AGCTGATTCC TTTTGTATT TGTTGGAAG
1380

TAATCAATAG TGGAGTCAAG AACAGGTTT GGTGTGAAGT AACGGGAGTG GTAGGAGAAG
1440

GGTTGGGGGA TTGTATGGCG GGAGGAGTAG TTTACATATG GGTCTAGGT TAGGGCTGTG
1500

GCCTTGTTA CAAAGTTATC ATCTAGAATA ACAGCAGTGG AGCCCACCTCC CCTATCACCC
1560

TGGGTGATGG GGGAGCAGGG CCAGAATTCA ACCTAACCT TTCTTATTCT GTAGTATTCA
1620

AAGGGTATAG AGATTTGTT GGTCCCCCT CCCGGGGAA CAAAGTCGTC AATTTAAAT
1680

CTCATCATGT CCACCGCCCA GGAGGGCGTT GTGACTGTGG TACGCTTGAC AGTATATCCG
1740

AAGGTGCGGG AGAGGCAGGT GTTGAAGATG CCATTTTCC TTCTCCAACG GTAGCGGTGG
1800

CGGGGGTGGG CGAGCCAGGG GCGGCGGCAGG AGGATCTGGC CAAGATGGCT GCGGGGGCGG
1860

TGTCTTCTTC TGCAGTAACG CCTCCTTGGG TACGTCTTAA CGATACAAAC TTAACGGATA
1920

TCGCGATAAT GAAATAATTG ATGATTATTT CTCGCTTCA ATTTAACACA ACCCTCAAGA
1980

ACCTTTGTAT TTATTTCAC TTTTTAAGTA TAGAATAAAAG AAGCTGGGGG ATCAATTCC
2040

GCAGCCCTGC AGCTAATTAA TTAAGCTACA AATAGTTCG TTTTCACCTT GTCTAATAAC
2100

TAATTAATTA AGGATCCCCC AGCTTCTTTA TTCTATACTT AAAAAGTGA AATAAAATACA
2160

AAGGTTCTTG AGGGTTGTGT TAAATTGAAA GCGAGAAATA ATCATAAAATT ATTTCATTAT
2220

CGCGATATCC GTTAAGTTTG TATCGTAATG CCCAGCAAGA AGAATGGAAG AAGCGGACCC
2280

CAACCACATA AAAGGTGGGT GTTCACGCTG AATAATCCTT CCGAAGACGA GCGCAAGAAA
2340

ATACGGGAGC TCCCAATCTC CCTATTTGAT TATTTTATTG TTGGCGAGGA GGGTAATGAG
2400

GAAGGACGAA CACCTCACCT CCAGGGGTTTC GCTAATTTG TGAAGAAGCA AACTTTAAT
2460

AAAGTGAAGT GGTATTTGGG TGCCCGCTGC CACATCGAGA AAGCCAAAGG AACTGATCAG
2520

CAGAATAAAG AATATTGCAG TAAAGAAGGC AACTTACTTA TTGAATGTGG AGCTCCTCGA

PAGE MISSING AT TIME OF PUBLICATION

TGATGATTAA AATGTGAACC GTCCAAATT GCAGTCATTA TATGAGCGTA TCTATTATCT
3480

ACTATCATCA TCTTGAGTT ATTAATATCA TCTACTTTAG AATTGATAGG AAATATGAAT
3540

ACCTTTGTAG TAATATCTAT ACTATCTACA CCTAACTCAT TAAGACTTT GATAGGCGGC
3600

CGCGAGCTC
3609

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 53 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATCATCATG ATATCCGTTA AGTTTGTATC GTAATGACGT GGCCAAGGAG GCG
53

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TACTACTACG TCGACTTATT TATTTAGAGG GTCTTTAGG

40

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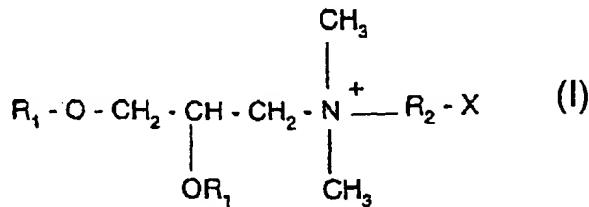
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(54) Title: DNA VACCINE - PCV



(57) Abstract: The invention relates to immunogenic preparations or vaccines comprising, on the one hand, a plasmid encoding and expressing a gene from PCV, in particular selected from the group consisting of ORF1 of PCV-2, ORF2 of PCV-2, ORF1 of PCV-1 and ORF2 of PCV-1, and, on the other hand, an element capable of increasing the immune response directed against the product of expression of the gene, which can be a carbohydrate, a porcine cytokine, e.g. GM-CSF or a cationic lipid of formula (I), in which R₁ is a saturated or unsaturated linear aliphatic radical having from 12 to 18 carbon atoms, R₂ is another aliphatic radical comprising from 2 to 3 carbon atoms, and X is a hydroxyl or amine group. The cationic lipid can be DMRIE, possibly coupled with DOPE.

DNA Vaccine - PCV

The present invention relates to plasmid 5 constructs encoding and expressing porcine circovirus (PCV for Porcine CircoVirus) immunogens responsible for the PMWS syndrome (Porcine Multisystemic Wasting Syndrome or Post-Weaning Multisystemic Wasting Syndrome), to methods of vaccination and to DNA 10 vaccines, as well as to methods of producing and of formulating these vaccines. All documents cited herein, and all documents cited in documents cited herein are hereby incorporated herein by reference.

PCV was originally detected as a 15 noncytopathogenic contaminant in pig kidney cell lines PK/15. This virus was classified among the *Circoviridae* with the chicken anaemia virus (CAV for Chicken Anaemia Virus) and the PBFDV virus (Pscittacine Beak and Feather Disease Virus). These are small nonenveloped 20 viruses (from 15 to 24 nm) whose common characteristic is to contain a genome in the form of a circular single-stranded DNA of 1.76 to 2.31 kilobases (kb). It was first thought that this genome encoded a polypeptide of about 30 kDa (Todd *et al.*, *Arch. Virol.*, 25 1991, **117**: 129-135). Recent work has however shown a more complex transcription (Meehan B.M. *et al.*, *J. Gen. Virol.*, 1997, **78**: 221-227). Moreover, no significant homologies in nucleotide sequence or in common 30 antigenic determinants are known between the three species of circoviruses known.

The PCV derived from PK/15 cells is considered not to be pathogenic. Its sequence is known from B.M. Meehan *et al.*, *J. Gen. Virol.* 1997 (**78**) 221-227. It is only very recently that some authors have thought 35 that strains of PCV could be pathogenic and associated with the PMWS syndrome (G.P.S. Nayar *et al.*, *Can. Vet. J.*, 1997, **38**: 385-387 and Clark E.G., *Proc. Am. Assoc. Swine Pract.* 1997: 499-501). Nayar *et al.* have detected PCV DNA in pigs having the PMWS syndrome using PCR 40 techniques.

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Monoclonal and polyclonal antibodies directed against circoviruses found in pigs having the symptoms of the PMWS syndrome have been able to demonstrate differences between these circoviruses and the porcine 5 circoviruses isolated from culture of PK-15 cells (Allan G.M. et al. Vet Microbiol., 1999, 66: 115-123).

The PMWS syndrome detected in Canada, the United States and France is clinically characterized by a gradual loss of weight and by manifestations such as 10 tachypnea, dyspnea and jaundice. From the pathological point of view, it is manifested by lymphocytic or granulomatous infiltrations, lymphadenopathies and, more rarely, by hepatitis and lymphocytic or granulomatous nephritis (Clark E. G., Proc. Am. Assoc. 15 Swine Prac. 1997: 499-501; La Semaine Vétérinaire No. 26, supplement to La Semaine Vétérinaire 1996 (834); La Semaine Vétérinaire 1997 (857): 54; G.P.S. Nayer et al., Can. Vet. J., 1997, 38: 385-387).

These circoviruses obtained from North America 20 and from Europe are very closely related, with a degree of identity of more than 96% of their nucleotide sequence, whereas the degree of identity is less than 80% when the nucleotide sequences of these circoviruses are compared with those of porcine circoviruses 25 isolated from PK-15 cells. Accordingly, two viral subgroups have been proposed, PCV-2 for the circoviruses associated with the PMWS syndrome and PCV-1 for the circoviruses isolated from the PK-15 cells (Meehan B.M. et al., J. Gen. Virol., 1998, 79: 30 2171-2179; WO-A-9918214).

The Applicant has found that plasmid constructs encoding and expressing PCV-2 immunogens can be used to immunize pigs against the PMWS syndrome.

PCV-2 immunogens can be used in combination 35 with PCV-1 immunogens to also immunize these animals against PCV-2.

According to a less preferred mode, the PCV-1 immunogens may be used alone.

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The subject of the present invention is plasmid constructs encoding and expressing a PCV-1 or PCV-2 immunogen, in particular the open reading frames (ORFs) 1 and/or 2 for PCV-1, and the ORFs 1 and/or 2 for PVC-2
5 (ORF means Open Reading Frame).

It goes without saying that the invention automatically covers the plasmids encoding and expressing equivalent nucleotide sequences, that is to say the sequences which change neither the 10 functionality or the strain specificity (say strain of type 1 and strain of type 2) of the gene considered or those of the polypeptides encoded by this gene. The sequences differing through the degeneracy of the code will, of course, be included.

15 The PCV-2 sequences used in the examples are derived from Meehan et al. *supra* (Strain Imp.1010 ; ORF1 nucleotides 398-1342; ORF2 nucleotides 1381-314; and correspond respectively to ORF4 and ORF13 in US 09/161,092 of 25 September 1998 and to COL4 and 20 COL13 in WO-A-9918214). Other PCV-2 strains and their sequences have been published in WO-A-9918214 and called Imp1008, Imp999, Imp1011-48285 and Imp1011-48121, as well as in A.L. Hamel et al. J. Virol. June 1998, vol 72, 6: 5262-5267 (GenBank 25 AF027217) and in I. Morozov et al. J. Clinical Microb. Sept. 1998 vol. 36, 9: 2535-2541, as well as GenBank AF086834, AF086835 and AF086836, and give access to equivalent ORF sequences.

30 The invention also covers the equivalent sequences in the sense that they are capable of hybridizing to the nucleotide sequence of the gene considered under high stringency conditions. Among the equivalent sequences, there may also be mentioned the gene fragments conserving the immunogenicity of the 35 complete sequence.

The homology of the whole genome of types 1 and 2 therebetween is about 75%. For ORF1, it is about 86%, and for ORF2, about 66%. On the contrary, homologies

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between genomes and between ORFs inside type 2 are generally above 95%.

Are also equivalent sequences according to the present invention, for ORF1, those sequences having an 5 homology equal or greater than 88%, in particular than 90%, preferably than 92% or 95% with ORF1 of strain Imp1010, and for ORF2, those sequences having an homology equal or greater than 80%, in particular than 85%, preferably than 90% or 95% with ORF2 of strain 10 Imp1010.

ORF1 and ORF2 according to Meehan 1998 has the potential to encode proteins with predicted molecular weights of 37.7 kD and 27.8 kD respectively. ORF3 and ORF4 (according to Meehan et al. 1998, correspond to 15 ORF7 and ORF10 respectively in WO-A-9918214) has the potential to encode proteins with predicted molecular weights of 11.9 and 6.5 kD respectively. The sequence of these ORFs is also available in Genbank AF 055392. They can also be incorporated in plasmids and be used i 20 accordance with the invention alone or in combination, e.g. with ORF1 and/or ORF2.

The other ORFs 1-3 and 5, 6, 8-9, 11-12 disclosed in US 09/161,092 of 25 September 1998 (COLs 1-3 and 5, 6, 8-9, 11-12 in WO-A-9918214), may be used 25 under the conditions described here, in combination or otherwise with each other or with the ORFs 1 and 2 as defined here.

This also encompasses the use of equivalent sequences in the leaning given above, in particular 30 those ORFs coming from various PCV-2 strains cited herein. For homology, one can precise that are equivalent those sequences which come from a PCV strain having an ORF2 and/or an ORF1 which have an homology as defined above with the corresponding ORF of strain 35 1010. For ORF3 according to Meehan, it can also be said that homology has to be for instance equal or greater than 80%, in particular than 85%, preferably than 90% or 95% with ORF3 of strain Imp1010. For ORF4 according to Meehan 1998, it can be equal or greater than 86%, in

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particular than 90%, preferably than 95% with ORF4 of strain Imp1010.

From the genomic nucleotide sequence, e.g. those disclosed in WO-A-99 18214, it is routine art to 5 determine the ORFs using a standard software, such as MacVector®. Also, alignment of genomes with that of strain 1010 and comparison with strain 1010 ORFs allows the one skilled in the art to readily determine the ORFs on the genome for another strain (e.g. those 10 disclosed in WO-A-99 18214). Using a software or making alignment is not undue experimentation and give directly access to equivalent ORFs.

The word plasmid is here intended to cover any DNA transcription unit in the form of a polynucleotide 15 sequence comprising the PCV sequence to be expressed and the elements necessary for its expression *in vivo*. The circular plasmid form, supercoiled or otherwise, is preferred. The linear form is also included within the scope of the invention.

20 The subject of the present invention is more particularly the plasmids called pJP109 (containing the ORF2 gene of PCV-2, Figure 1), pJP111 (containing the ORF1 gene of PCV-2, Figure 2), pJP120 (containing the ORF2 gene of PCV-1, Figure 3) and pJP121 (containing 25 the ORF1 gene of PCV-1, Figure 4).

Each plasmid comprises a promoter capable of ensuring, in the host cells, the expression of the inserted gene under its control. It is in general a strong eukaryotic promoter and in particular a 30 cytomegalovirus early promoter CMV-IE, of human or murine origin, or optionally of other origin such as rat or guinea pig. More generally, the promoter is either of viral origin or of cellular origin. As a viral promoter other than CMV-IE, there may be 35 mentioned the SV40 virus early or late promoter or the Rous Sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the promoter specific to the gene. As cellular promoter, there may be mentioned the promoter

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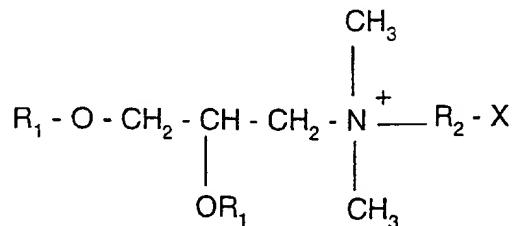
of a cytoskeleton gene, such as for example the desmin promoter, or alternatively the actin promoter. When several genes are present in the same plasmid, they may be provided in the same transcription unit or in two
5 different units.

The plasmids may also comprise other transcription regulating elements such as, for example, stabilizing sequences of the intron type, preferably intron II of the rabbit β -globin gene (van Ooyen et al.
10 Science, 1979, 206: 337-344), signal sequence of the protein encoded by the tissue plasminogen activator gene (tPA; Montgomery et al. Cell. Mol. Biol. 1997, 43: 285-292), and the polyadenylation signal (polyA), in particular of the bovine growth hormone (bGH) gene
15 (US-A-5,122,458) or of the rabbit β -globin gene.

The subject of the present invention is also immunogenic preparations and DNA vaccines comprising at least one plasmid according to the invention, encoding and expressing one of the PCV-1 or PCV-2 immunogens,
20 preferably one of the abovementioned ORFs, in addition a veterinarilly acceptable vehicle or diluent, with optionally, in addition, a veterinarilly acceptable adjuvant.

The subject of the present invention is more particularly immunogenic preparations and vaccines containing at least one plasmid encoding and expressing one of the PCV-1 or PCV-2 immunogens, compositions formulated with an adjuvant, in particular a cationic
25 lipid containing a quaternary ammonium salt of formula

30



in which R_1 is a saturated or unsaturated linear aliphatic radical having from 12 to 18 carbon atoms, R_2

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is another aliphatic radical comprising from 2 to 3 carbon atoms, andt X is an hydroxyle ou amine group.

Preferably it is DMRIE (N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanammonium; 5 WO-A-9634109), and preferably coupled with a neutral lipid, e.g. preferably DOPE (dioleoylphosphatidylethanolamine), to form DMRIE-DOPE. Preferably, the plasmid mixture with this adjuvant is made immediately before use and preferably, before its 10 administration to the animal, the mixture thus produced is allowed to form a complex, for example over a period ranging from 10 to 60 minutes, in particular of the order of 30 minutes.

When DOPE is present, the DMRIE:DOPE molar 15 ratio preferably ranges from 95:5 to 5:95, more particularly 1:1.

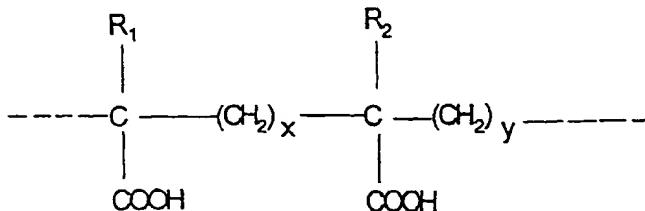
The plasmid:DMRIE or DMRIE-DOPE adjuvant weight ratio may range in particular from 50:1 to 1:10, in particular from 10:1 to 1:5, preferably from 1:1 to 20 1:2.

According to another advantageous mode of the invention, it is possible to use, as adjuvant, an adjuvant compound selected from the polymers of acrylic or methacrylic acid and the copolymers of maleic 25 anhydride and of alkenyl derivative. The polymers of acrylic or methacrylic acid crosslinked in particular with polyalkenyl ethers of sugars or of polyalcohols are preferred. These compounds are known by the term carbomer (Pharmeuropa vol. 8, No. 2, June 1996). 30 Persons skilled in the art can also refer to US-A-2,909,462 (incorporated by reference) describing such acrylic polymers crosslinked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms 35 of at least three hydroxyls being replaced with unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated

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radicals may themselves contain other substituents, such as methyl. The products sold under the name Carbopol® (GF Goodrich, Ohio, USA) are particularly appropriate. They are crosslinked with an allyl 5 saccharose or with allylpentaerythritol. Among them, there may be mentioned Carbopol® 974P, 934P and 971P.

Among the copolymers of maleic anhydride and of an alkenyl derivative, the EMAs® (Monsanto) are preferred which are copolymers of maleic anhydride and 10 ethylene, linear or crosslinked, for example crosslinked with divinyl ether. Reference may be made to J. Fields *et al.* Nature, 186 : 778-780, 4 June 1960 (incorporated by reference). From the point of view of their structure, the polymers of acrylic or methacrylic 15 acid and the EMAs® preferably consist of basic units of the following formula:



in which

20 - R_1 and R_2 , which are identical or different, represent H or CH_3
 - $x = 0$ or 1, preferably $x = 1$
 - $y = 1$ or 2, with $x + y = 2$

For the EMAs®, $x = 0$ and $y = 2$. For the 25 carboomers, $x = y = 1$.

The dissolution of these polymers in water leads to an acidic solution which will be neutralized, preferably to physiological pH, to give the adjuvant solution into which the actual vaccine will be 30 incorporated. The carboxyl groups of the polymer are then partly in COO^- form.

For this type of adjuvant, it is preferable to prepare a solution of the adjuvant, in particular of carboomer, in distilled water, preferably in the 35 presence of sodium chloride, the solution obtained

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being at acidic pH. This stock solution is diluted by adding it to the required quantity (in order to obtain the desired final concentration), or a substantial part thereof, of water loaded with NaCl, preferably 5 physiological saline (NaCl 9 g/l), in one or more portions with concomitant or subsequent neutralization (pH 7.3 to 7.4), preferably with NaOH. This solution at physiological pH will be used as it is to mix with the plasmid, in particular stored in lyophilized, 10 liquid or frozen form.

The polymer concentration in the final vaccine composition will be 0.01% to 2% w/v, more particularly 0.06 to 1% w/v, preferably 0.1 to 0.6% w/v.

15 In a specific embodiment, the immunogenic or vaccine preparation comprises a plasmid or a mixture of plasmids encoding and expressing PCV-2 ORF1 and ORF2.

The invention also provides for combining the vaccination against the porcine circovirus with a vaccination against other pig pathogens, in particular 20 those which may be associated with the PMWS syndrome. By way of example, one may cite : Aujeszky's disease virus, porcine influenza virus, PRRS, porcine parvovirus, hog cholera virus, *Actinobacillus pleuropneumoniae*.

25 The subject of the present invention is thus mixtures of plasmid containing at least one plasmid according to the invention and at least another plasmid encoding and expressing a porcine immunogen, selected for example from the group consisting of the 30 glycoproteins gB and gD of the Aujeszky's disease virus (pseudorabies virus or PRV), the haemagglutinin and the nucleoprotein of the porcine influenza virus H1N1, the haemagglutinin and the nucleoprotein of the porcine influenza virus H3N2, the ORF5 and ORF3 genes of the 35 PRRS virus of the Lelystad and USA strains, the VP2 protein of the porcine parvovirus, the E1 and E2 proteins of the hog cholera virus (HCV), the deleted apxI, apxII and apxIII genes from *Actinobacillus*

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pleuropneumoniae (see for the plasmids for example WO-A-9803658).

These mixtures of plasmids are taken up in a 5 veterinarily acceptable vehicle or diluent, with optionally, in addition, a veterinarily acceptable adjuvant as described above, thus forming immunogenic preparations or multivalent DNA vaccines. These 10 preparations or multivalent vaccines may in particular be advantageously formulated with a cationic lipid as described above, in particular DMRIE, and preferably coupled with a neutral lipid, DOPE, to form the DMRIE-DOPE.

The preparations or monovalent or multivalent 15 DNA vaccines according to the invention, formulated or otherwise with an adjuvant as described above, may also be advantageously supplemented with a cytokine preferably of porcine origin, in particular porcine GM-CSF. This addition of porcine GM-CSF (granulocyte 20 macrophage - colony stimulating factor; Clark S.C. et al. Science 1987, 230: 1229; Grant S.M. et al. Drugs, 1992, 53: 516) may be carried out in particular by incorporating into the preparation or into the vaccine either porcine GM-CSF protein, or a plasmid encoding 25 and expressing the porcine GM-CSF gene (Inumaru S. and Takamatsu H. Immunol. Cell. Biol., 1995, 73: 474-476). Preferably, the porcine GM-CSF gene is inserted into a plasmid different from those encoding the PCV immunogens or the other porcine immunogens.

In particular, the plasmid encoding and 30 expressing the porcine GM-CSF may be the plasmid pJP058 (Figure 5).

The immunogenic preparations and the monovalent or multivalent DNA vaccines according to the invention may also be combined with at least one conventional 35 vaccine (attenuated live, inactivated or subunit) or recombinant vaccine (viral vector) directed against at least one porcine pathogen which is different or identical. The invention provides in particular for the combination with adjuvant-containing conventional

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vaccines (attenuated live, inactivated or subunit). For the inactivated or subunit vaccines, there may be mentioned those containing in particular alumina gel alone or mixed with saponin as adjuvant, or those 5 formulated in the form of an oil-in-water emulsion.

The subject of the present invention is also a method of immunization which makes it possible to induce an immune response in pigs towards the circoviruses according to the invention. Its subject is 10 in particular a method of vaccination which is effective in pigs. These methods of immunization and vaccination comprise the administration of one of the preparations or of one of the monovalent or multivalent DNA vaccines as described above. These methods of 15 immunization and vaccination comprise the administration of one or more successive doses of these preparations or DNA vaccines. The preparations and DNA vaccines may be administered, in the context of this method of immunization or of vaccination, by various 20 routes of administration proposed in the prior art for polynucleotide vaccination, in particular the intramuscular and intradermal routes, and by means of known administration techniques, in particular injections with a syringe having a needle, by liquid 25 jet (Furth et al. Analytical Bioch., 1992, 205: 365-368) or by projection of gold particles coated with DNA (Tang et al. Nature, 1992, 356: 152-154).

This method not only allows for administration to adult pigs, but also to the young and to gestating 30 females; in the latter case, this makes it possible, in particular, to confer passive immunity onto the newborns (maternal antibodies). Preferably, female pigs are inoculated prior to breeding; and/or prior to serving, and/or during gestation. Advantageously, at 35 least one inoculation is done before serving and it is

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preferably followed by an inoculation to be performed during gestation, e.g., at about mid-gestation (at about 6-8 weeks of gestation) and/or at the end of gestation (at about 11-13 weeks of gestation). Thus, an 5 advantageous regimen is an inoculation before serving and a booster inoculation during gestation. Thereafter, there can be reinoculation before each serving and/or during gestation at about mid-gestation and/or at the end of gestation. Preferably, reinoculations are during 10 gestation.

Piglets, such as piglets from vaccinated females (e.g., inoculated as herein discussed), are inoculated within the first weeks of life, e.g., inoculation at one and/or two and/or three and/or four and/or five 15 weeks of life. Preferably, piglets are first inoculated within the first week of life or within the third week of life (e.g., at the time of weaning). Advantageously, such piglets are then boosted two to four weeks later.

The quantity of DNA used in the vaccines 20 according to the present invention is between about 10 µg and about 2000 µg, and preferably between about 50 µg and about 1000 µg. Persons skilled in the art will have the competence necessary to precisely define the effective dose of DNA to be used for each 25 immunization or vaccination protocol.

The dose volumes may be between 0.5 and 5 ml, preferably between 2 and 3 ml.

A preferred method of immunization or of 30 vaccination consists in the administration of the DNA vaccines according to the invention by the intramuscular route.

The invention will now be described in greater detail with the aid of nonlimiting exemplary embodiments, taken with reference to the drawing, in 35 which:

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Figure 1: plasmid pJP109
Figure 2: plasmid pJP111
Figure 3: plasmid pJP120
Figure 4: plasmid pJP121
5 Figure 5: plasmid pJP058

Sequence listing SEQ ID

SEQ ID No. 1: oligonucleotide JP779
SEQ ID No. 2: oligonucleotide JP780
10 SEQ ID No. 3: oligonucleotide JP781
SEQ ID No. 4: oligonucleotide JP782
SEQ ID No. 5: oligonucleotide JP783
SEQ ID No. 6: oligonucleotide JP784
SEQ ID No. 7: oligonucleotide JP785
15 SEQ ID No. 8: oligonucleotide JP786
SEQ ID No. 9: oligonucleotide RG972
SEQ ID No. 10: oligonucleotide RG973

EXAMPLES

20 PCV-2 strains useful for cloning ORFs are for instance strains deposited at the ECACC and having the accession numbers V97100219 (Imp1008), V97100218 (Imp1010) and V97100217 (Imp999) (which were deposited on October 2, 1997), V98011608 (Imp1011-48285) and V98011609 (Imp1011-48121) (which were deposited on January 16, 1998).
25 These examples are constructed using strain Imp1010. The one skilled in the art is able to adapt the process to other PCV-2 strains.

30

Example 1 Construction of the plasmid pJP109

The plasmid pGEM7Z-Imp1010 Stoen-EcoRI No. 14 containing the genome of the PCV-2 virus in the form of an EcoRI fragment (B. Meehan et al. J. Gen. Virol. 35 1998. 79 2171-2179) was digested with EcoRI in order to isolate, after agarose gel electrophoresis, the EcoRI-EcoRI fragment of 1768 base pairs (bp). This fragment was self-ligated.

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The ORF2 gene of the PCV-2 virus strain 1010-Stoon (B. Meehan et al. J. Gen. Virol. 1998. 79. 2171-2179; GenBank sequence accession No. AF055392) was amplified, using the template consisting of the self-ligated 5 EcoRI-EcoRI fragment, by the polymerase chain reaction (PCR) technique with the following oligonucleotides:

JP779 (SEQ ID NO 1) (35 mer):

5'CATCATCATGTCGACATGACGTATCCAAGGAGGCG3'

and JP780 (SEQ ID NO 2) (36 mer):

10 5'TACTACTACAGATCTTAGGGTTAACGTGGGGGGTC3'

in order to generate a 730 bp PCR fragment. This fragment was digested with SalI and BglII in order to isolate, after agarose gel electrophoresis, the 715 bp SalI-BglII restriction fragment. This fragment was then 15 ligated with the plasmid pVR1012 (Hartikka J. et al. Human Gene Therapy. 1996. 7. 1205-1217), digested beforehand with SalI and BglII, to give the plasmid pJP109 (5567 pb) (Figure 1).

20 **Example 2: Construction of the plasmid pJP111**

A polymerase chain reaction was carried out with the plasmid pGem7Z-Imp1010-Stoon (see Example 1) (B. Meehan et al. J. Gen. Virol. 1998. 79. 2171-2179), and the following oligonucleotides:

25 JP781 (SEQ ID NO 3) (35 mer):

5'CATCATCATGTCGACATGCCAGCAAGAAGAATGG3'

and JP782 (SEQ ID NO 4) (36 mer):

5'TACTACTACAGATCTCAGTAATTATTCATATGG3'

in order to generate a 970 bp PCR fragment containing 30 the ORF1 gene of the PCV-2 virus. This fragment was digested with SalI and BglII in order to isolate, after agarose gel electrophoresis, the 955 bp SalI-BglII restriction fragment. This fragment was then ligated with the plasmid pVR1012 (Example 1) to give the 35 plasmid pJP111 (5810 bp) (Figure 2).

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Example 3: Construction of the plasmid pJP120 (PCV-1 ORF2)

A polymerase chain reaction was carried out with the plasmid pPCV1 (B. Meehan et al. J. Gen. Virol. 1997.

5 78, 221-227), and the following oligonucleotides;

JP783 (SEQ ID NO 5) (35 mer):

5'CATCATCATGTCGACATGACGTGGCCAAGGAGGCG3'

and JP784 (SEQ ID NO 6) (40 mer):

5'TACTACTACAGATCTTATTAGAGGGTCTTTAGG3'

10 in order to generate a 730 bp PCR fragment containing the ORF2 gene of the PCV-1 virus (PK-15 strain, GenBank sequence accession No. U49186). This fragment was digested with SalI and BglII in order to isolate, after agarose gel electrophoresis, the 715 bp SalI-BglII 15 restriction fragment. This fragment was then ligated with the plasmid pVR1012 (Example 1) to give the plasmid pJP120 (5565 bp) (Figure 3).

Example 4: Construction of the plasmid pJP121 (PCV-1 20 ORF1)

The plasmid pPCV1 containing the PCV1 virus genome in the form of a PstI fragment (B. Meehan et al. J. Gen. Virol. 1997, 78, 221-227) was digested with PstI in order to isolate, after agarose gel electrophoresis, 25 the 1759 base pair (bp) PstI-PstI fragment. This fragment was self-ligated.

The ORF1 gene of the PCV-1 virus strain PK-15 (B. Meehan et al. J. Gen. Virol. 1997, 78, 221-227; GenBank sequence accession No. U49186) was amplified, 30 using the template consisting of the self-ligated PstI-PstI fragment, by the polymerase chain reaction (PCR) technique with the following oligonucleotides:

JP785 (SEQ ID NO 7) (35 mer):

5'CATCATCATGTCGACATGCCAAGCAAGAAAAGCGG3'

35 and JP786 (SEQ ID NO 8) (36 mer):

5'TACTACTACAGATCTTCAGTAATTATTTATATGG3'

in order to generate a 965 bp PCR fragment containing the ORF1 gene of the PCV-1 virus (strain PK-15). This fragment was digested with SalI and BglII in order to

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isolate, after agarose gel electrophoresis, the 946 bp SalI-BglII restriction fragment. This fragment was then ligated with the plasmid pVR1012 (Example 1) to give the plasmid pJP121 (5804 bp) (Figure 4).

5

Example 5: Construction of the plasmid pJP058 (expressing porcine GM-CSF)

Pig blood was collected over a tube containing EDTA by taking blood from the jugular vein. The mononucleated 10 cells were harvested by centrifugation on a Ficoll gradient and then cultured *in vitro* in RPMI 1640 medium (Gibco-BRL) and stimulated by addition of concanavaline A (Sigma) at a final concentration of about 5 µg/ml in the culture medium. After 72 hours of stimulation, the 15 lymphoblasts were harvested and the total RNA of these cells was extracted with the extraction kit "Micro-Scale Total RNA Separator Kit" (Clontech) following the manufacturer's recommendations. A reverse transcription reaction, carried out with the aid of the kit 20 "1st-Strand cDNA Synthesis Kit" (Perkin Elmer), followed by a polymerase chain reaction, was carried out on the total RNA extracted from these porcine lymphoblasts with the following oligonucleotides:

25 RG972 (33 mer): (SEQ ID No. 9)

5' TATGCGGCCGCCACCATGTGGCTGCAGAACCTG3'

and RG973 (34 mer): (SEQ ID No. 10)

5' TATGCGGCCGCTACGTATCACTTCTGGGCTGGTT3'

in order to generate a PCR fragment of about 450 base 30 pairs (bp). This fragment was digested with NotI in order to isolate, after agarose gel electrophoresis, the 450 bp NotI-NotI fragment. This fragment was then ligated with the plasmid pVR1012 (Example 1), preferably digested with NotI and dephosphorylated, to 35 give the plasmid pJP058 (5405 bp) (Figure 5). The sequence of the pGM-CSF gene cloned into the plasmid pJP058 was checked and found to be identical to that available in the GenBank database (accession No. D21074).

Example 6: Production of the purified plasmids for the vaccination of pigs

Escherichia coli K12 bacteria (strains DH10B or SCS1) 5 were transformed with the plasmids pJP109, pJP111, pJP058, pJP120 and pJP121 of Examples 1 to 5 *supra*. The five transformed clones obtained respectively with these five plasmids were then cultured separately, with shaking at +37°C, in Luria-Broth (LB) medium. The 10 bacterial cultures were harvested at the end of the exponential phase and the plasmids were extracted according to the alkaline lysis technique. The extracted plasmids were then purified on a caesium chloride gradient according to the technique described 15 by Sambrook et al. (Molecular Biology: A Laboratory Manual, 2nd Edition, 1989, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). After final extraction of ethidium bromide and precipitation in the presence of absolute ethanol, the purified plasmids 20 were resuspended in TE buffer (1 mM Tris/EDTA, pH 8.0) in order to obtain stock solutions containing 2 mg of plasmid per ml. These stock solutions are stored at -20°C before use.

25 **Example 7: Control of the expression of ORFs 1 and 2 of the PCV-2 virus**

In order to control the products of expression of the PCV-2 ORF2 and PCV-2 ORF1 genes, cloned respectively 30 into the plasmids pJP109 and pJP111, these plasmids were transfected into CHO-K1 (Chinese Hamster Ovary) cells (ATCC No. CCL-61) with the Lipofectamine Plus® 35 transfection kit (Gibco-BRL, Catalogue# 10964-013), following the manufacturer's recommendations for use. 48 hours after transfection, the transfected cells are washed and fixed with a 95% glacial acetone solution 35 for 3 minutes at room temperature. Five monoclonal antibodies specific for the PCV-2 ORF1 proteins (F199 1D3GA and F210 7G5GD) and ORF2 proteins (F190 4C7CF, F190 2B1BC and F190 3A8BC) were used as

- 18 -

first antibodies. An anti-mouse IgG conjugate, labelled with Cy3, was used to reveal the specific labelling. A PCV-2 specific fluorescence was observed with the 3 PCV-2 ORF2 monoclonals in the cells transfected with 5 the plasmid pJP109, but not in those transfected with the plasmid pJP111. In contrast, a PCV-2 specific fluorescence was observed with the two PCV-2 ORF1 monoclonals in the cells transfected with the plasmid pJP111, but not in those transfected with the plasmid 10 pJP109. No fluorescence was detected with the PCV-2 monoclonals in CHO cells transfected with the plasmid pVR1012 alone or in the nontransfected CHO cells.

The same expression result was obtained with a polyclonal serum specific for the PCV-2 virus. In this 15 case, a fluorescein-labelled anti-pig IgG conjugate was used to detect the specific fluorescence. No fluorescence was detected with this polyclonal serum in CHO cells transfected with the plasmid pVR1012 alone or in the nontransfected CHO cells.

20

Example 8: Vaccination of pigs with naked DNA

8.1. One-day-old piglets

25 Groups of piglets obtained by Caesarean on D0 of the protocol, are placed in an isolating unit. These piglets are vaccinated at the age of 2 days by the intramuscular route with various vaccinal solutions of plasmid. The vaccinal solutions are prepared by 30 diluting the stock solutions in sterile physiological saline (0.9% NaCl).

The piglets are vaccinated:
either with the plasmid pJP109 alone
or with the mixture of the plasmids pJP109 and pJP111
35 or with the mixture of the plasmids pJP109 and pJP058
or with the mixture of the plasmids pJP109, pJP111 and pJP058
The vaccinal solutions comprise 500 µg of each plasmid.

- 19 -

Volume: The vaccinal solutions are injected by the intramuscular route in a total volume of 2 ml. In practice, given the age of the piglets on vaccination (1-2 days), 1 injection of 1 ml is given on each side 5 of the neck (= 2 x 1 ml).

Two injections of vaccine are carried out at two weeks' interval, that is to say on days D2 and D14 of the protocol.

A challenge is made on D21 of the protocol by oronasal 10 administration of a viral suspension of a virulent PCV-2 strain. The piglets are then monitored for 3 weeks for the appearance of specific clinical signs of post-weaning multisystemic wasting syndrome in piglets. The signs which are monitored are:

15 rectal temperature: daily measurement for the first 14 days, then two measurements during the 3rd week following the challenge.

Weight: weighing of the piglets just before the challenge then once per week during the 3 weeks 20 following the challenge.

Collection of blood samples to test for viremia and 25 antibodies: blood samples taken on D2, D14, D21, D28, D35 and D42.

Autopsy: on D42, the surviving pigs are humanely killed 25 and undergo autopsy to search for anatomicopathological lesions and to make histological preparations from the liver, the lymph nodes, the spleen, the kidneys and the thymus to search for lesions in these tissues.

30 **8.2. 5-7-week old piglets**

5- to 7-week old piglets, no longer having maternal antibodies specific for the PCV-2 virus are vaccinated by the intramuscular route:

either with the plasmid pJP109 alone,

35 or with the mixture of the plasmids pJP109 and pJP111
or with the mixture of the plasmids pJP109 and pJP058
or with the mixture of the plasmids pJP109, pJP111 and pJP058

- 20 -

the vaccinal doses are the same as those indicated in Example 8.1 (500 µg per plasmid). The vaccinal solutions are injected by the intramuscular route in a volume of 2 ml (a single administration of 2 ml, into 5 the neck muscles).

Two vaccinations are performed at 21 days' interval (D0 and D21). A challenge is made 14 days after the last vaccination (D35) by intramuscular administration of a viral suspension of a virulent PCV-2 strain.
10 The pigs are then monitored for 8 weeks for the occurrence of specific clinical signs of the post-weaning multisystemic wasting syndrome in piglets. The clinical monitoring of the piglets after the challenge is identical to that described in Example 8.1 except
15 that the total duration of observation is this time 8 weeks.

Example 9: Vaccination of pigs with DNA formulated with DMRIE-DOPE

20 It is possible to use, in place of the naked plasmid DNA solutions described in Example 8, solutions of plasmid DNA formulated with DMRIE-DOPE. A DNA solution (containing one or more plasmids according to Example 6) at 1 mg/ml is prepared in 0.9% NaCl. A DMRIE-DOPE 25 solution at 0.75 mM is prepared by taking up a lyophilisate of DMRIE-DOPE in a suitable volume of sterile distilled water.

The formation of the plasmid DNA-cationic lipid complexes is achieved by diluting, in equal parts, the 30 DMRIE-DOPE solution at 0.75 mM with the DNA solution at 1 mg/ml in 0.9% NaCl. The DNA solution is introduced gradually, with the aid of a syringe mounted with a 26G needle, along the wall of the vial containing the cationic lipid solution so as to avoid the formation of 35 foam. Gentle shaking is carried out as soon as the two solutions have been mixed. A composition comprising 0.375 mM DMRIE-DOPE and 500 µg/ml of DNA is finally obtained.

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It is desirable for all the solutions used to be at room temperature for all the operations described above. The DNA/DMRIE-DOPE complex formation is performed at room temperature for 30 minutes before 5 immunizing the pigs.

The pigs are then vaccinated according to the conditions described in Examples 8.1. and 8.2.

Example 10 : Vaccination of piglets and results

10 **1st experiment :**

Groups of 3 or 4 piglets, caesarian-derived day 0 are placed into isolators. The piglets are vaccinated day 2 either with pJP109 alone or with pJP109 and pJP111 plasmids mixture and with a physiological solution for 15 the control group. Each plasmid is diluted in sterile physiological solution (NaCl 0,9%) at 250 µg/µl final concentration. A 2 ml volume is injected by intramuscular route in two points of 1 ml (1 point each side of the neck). A second injection of vaccine or 20 placebo is administered day 14. Vaccination with DNA is well tolerated by piglets and no evidence for adverse reaction to vaccination is noted. The piglets are challenged day 21 by oronasal administration of PCV-2 viral suspension, 1 ml in each nostril. After challenge 25 piglets are weighed once a week. Rectal temperatures are recorded on days 17, 21, 22, 24, 27, 29, 31, 34, 37, 41, 44. Day 44 fecal swabs are collected from each piglet for PCV-2 shedding. The virus is detected and quantified by quantitative PCR. Day 45 necropsies are 30 performed and tissue samples are collected for virus isolation.

• Clinical symptoms :

There is no significant difference for the mean body 35 weight gains or the mean body temperatures between groups.

- Necropsy lesions :

5 The only gross finding noted in pigs at termination is bronchial lymphadenopathy. The lesions are scored according the following criteria.

10 0 = no visible enlargement of lymph nodes

15 1 = mild lymph nodes enlargement, restricted to bronchial lymph nodes

20 2 = moderate lymph nodes enlargement, restricted to bronchial lymph nodes

25 3 = severe lymph nodes enlargement, extended to bronchial submandibular prescapular and inguinal lymph nodes.

30 std is an abbreviation for standard deviation
15 N is for number of animals in each group

Groups	Lymphadenopathy scores		
	mean	std	N
20 pJP109	1.2	1.3	4
25 pJP109 + pJP111	2.0	1.7	3
30 controls	3.0	0.0	3

35 N = number of piglets in each group
A reduction of the lymph node lesions is observed in 3 out 4 piglets immunized with pJP109 and 1 out 3 piglets immunized with pJP109 and pJP111 plasmids mixture. This difference is not significant ($p>0.05$) due to the high value of the standard deviations (std).

- Virus load in lymph nodes tissues:

35 Quantitative virus re-isolation is performed on tissue homogenates prepared from bronchial and mesenteric lymph nodes.

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The data presented correspond to the virus titers in tissue homogenates after transformation in \log_{10} .

5

PCV-2 titers

Groups	Bronchial LN		Mesenteric LN		
	mean	Std	mean	std	N
PJP109	0.9	0.8	0.9	0.8	4
PJP109 +	0.7	0.6	0.2	0.2	3
pJP111					
Controls	2.0	1.1	1.8	1.1	4

Bronchial lymph nodes seem to contain the most infectious virus. A reduction of the viral load is observed in bronchial and mesenteric lymph nodes from 10 piglets immunized with either pJP109 or pJP109 + pJP111 plasmids mixture. This reduction is significant ($p \leq 0.05$ for the plasmids mixture).

• Viral excretion:

15 Post challenge fecal swabs are assessed for shedding PCV-2 by PCR based on amplification of PCV-2 orf2. Each assay is performed in triplicate on 2 ml of sample. Unvaccinated controls are negative for PCV-2 prior challenge and positive after challenge 20 confirming the validity of the PCR assay.

Value are expressed as \log_{10} (number of molecules of PCV-2 DNA in 2 μ l sample).

Log₁₀ number of PCV-2 DNA molecules

	Groups	mean	std	N
5	pJP109	3.3	0.3	4
	pJP109 + pJP111	2.9	0.7	3
	Controls	3.6	0.6	4

The differences between groups are not significant (p
10 > 0.05).

2nd experiment :

14 day-old conventional piglets (8 per group) are
immunized with 2 administrations of the pJP109 and
15 pJP111 plasmids mixture formulated with DMRIE DOPE day
0 and day 20. For each administration 2 ml are injected
by intramuscular route on the side of the neck behind
the ear. The vaccine composition is 250 µg for each
plasmid /ml of physiological solution (0,9% NaCl) and
20 0.375 mM DMRIE DOPE.

For control group piglets are injected with the
physiological solution.

Day 32 the piglets are challenged by oronasal route,
25 introducing 5 ml of PCV-2 viral suspension at a 10^{5.8}
TCID50/ml titer with a syringe in each nostril.

The piglets are monitored for clinical symptoms,
prostration, vomiting, dyspnea, cough, anorexia and
30 hyperthermia (rectal temperature is recorded every day
during 28 days post challenge) slower growth (piglets
are weighed days 32, 40, 46, 53, 60). The symptoms are
scored according the following criteria: Annex1 (The
score for one piglet is equal to the sum of the scores
35 corresponding to the different days of observation)

- 25 -

Day 60 necropsies are performed and the lesions are scored according the following criteria: Annex2 (The score for one piglet is equal to the sum of the scores corresponding to each organ observed)

Tissue samples are collected, in particular lymph nodes.

10 Rectal swabs are collected days 32, 39, 42, 46, 49, 53, 56, 60 to follow viral excretion.

- Clinical symptoms:

15 A significant reduction of the clinical symptoms is observed in the group of immunized piglets compared to controls. In the control group 1 piglet died with PMWS symptoms and none in the vaccinated group.

20	Groups	Clinical scores		
		mean	std	N
	Vaccinated	13.5	7.1	8
	Controls	29.3	15.6	8

25 (p < 0.01 Kruskal-Wallis test)

A significant reduction of the duration of the post challenge hyperthermia is observed in the group of immunized piglet (p < 0.05).

30	Duration (days) of rectal temperature $\geq 40^{\circ}\text{C}$	Groups	mean	std	N
		Vaccinated	1.9	2.0	8
		Controls	8.4	3.9	8

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The daily weight gain post challenge is not significantly different between vaccinated and control groups.

5 • Necropsy lesions:

A significant reduction of the lesions is observed in the immunized piglets compared to controls in particular for lymphadenopathy ($p \leq 0.05$).

10 Global lesions and lymphadenopathy scores

	Groups	mean	std	N
Global lesions				
	Vaccinated	7.6	3.3	8
15	Controls	13.1	7.5	8

15 Lymph node scores

20	Vaccinated	3.1	2.7	8
	Controls	5.7	2.9	8

• Virus load in lymph nodes tissues:

The virus load in mesenteric and mediastinal lymph nodes is determined by immunochemistry.

25

The following criteria is used for the scores :

- 0 = lack of fluorescence
- 1 = some fluorescent foci on some organ slides
- 2 = approximately 1 foci per shot
- 30 - 3 = wholly fluorescent organ.

A significant reduction of the virus load is observed in the immunized groups ($p \leq 0.05$).

Groups	virus load			
	Mesenteric LN		Mediastinal LN N	
	mean	std	mean	std
5				
Vaccinated	0.5	0.6	1.3	0.2
Controls	1.8	0.8	2.0	0.8

• Viral excretion

10 The faecal swabs are assessed by PCR for PCV-2 shedding. The results are scored according the following criteria :

0 = absence of PCV-2

1 = presence of PCV-2

15 In the immunized group 38% of the piglets versus 88% in the control group excrete PCV-2 in the feces. The duration of viral excretion is significantly reduced in vaccinated group compared to controls.

20

Mean duration of viral excretion
(days)

Groups	mean	std	N
25 Vaccinated	1.2	2.1	8
Controls	11.4	6.3	8

30 It should be clearly understood that the invention defined by the appended claims is not limited to the specific embodiments indicated in the description above, but encompasses the variants which depart from neither the scope nor the spirit of the present invention.

ANNEXE 1 : Scores for clinical signs

signs	score
Prostration	0 no, 1 yes ; 2 can't get up
vomiting	0 no, 1 yes
dyspnea	0 no, 1 moderate ; 2 hight
cough	0 no, 1 yes
anorexia	0 no, 1 yes
hyperthermia	0 no, 1 $\geq 40^{\circ}\text{C}$; 2 $\geq 41^{\circ}\text{C}$
growing	0 no, 1 DWG week $x \leq$ DWG week $x-1$ and > 100 grams per day 2 DWG of the week ≤ 100 grams per day
death	0 no, x score of day just before the death

for a day the score is the sum of the score of each sign

ANNEXE 2 : Scores for macroscopic lesions

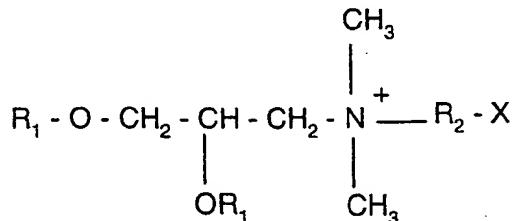
skin (color)	normal	0
	white	1
	yellow	2
corpulence	normal	0
	thin	1
	very thin	2
	cachectie	3
mucous	normal	0
	white	1
	yellow	2
sub. cut. Conjonctif	normal	0
	brillant	1
	yellow	2
ganglions (gg)	normal	0
	I large and or congestive	1
	> I large and or congestive	2
	> I very large	3
thoracic fluide	normal	0
	brillant	1
	visible	2
heart	normal	0
	lesion	1
lungs	normal	0
	lesion \leq 4	1
	lesion $> 4 \leq 6$	2
	lesion > 6	3
pleura	normal	0
	lesion	1
ascite	normal	0
	brillant	1
	visible	2
peritoneum	normal	0
	lesion	1
stomach	normal	0
	lesion	1
	ulcer	2
small intestine	normal	0
	lesion	1
large intestine	normal	0
	lesion	1
Peyers plaques	normal	0
	visible on 1 part of the intestine	1
	visible on 2 part of the intestine	2
	very importante	3
liver	normal	0
	lesion	1
kidney	normal	0
	lesion	1
bladder	normal	0
	lesion	1

CLAIMS

1. Immunogenic preparation or vaccine comprising,
 5 on the one hand, a plasmid encoding and expressing a
 gene selected from the group consisting of ORF1 of
 PCV-2, ORF2 of PCV-2, ORF1 of PCV-1 and ORF2 of PCV-1,
 and, on the other hand, an element capable of
 increasing the immune response directed against the
 10 product of expression of the gene.

2. Immunogenic preparation or vaccine according to
 Claim 1, characterized in that the element capable of
 increasing the immune response comprises as adjuvant a
 cationic lipid of formula

15



in which R_1 is a saturated or unsaturated linear
 aliphatic radical having from 12 to 18 carbon atoms, R_2
 is another aliphatic radical comprising from 2 to 3
 20 carbon atoms, and X is an hydroxyl or amine group.

3. Immunogenic preparation or vaccine according to
 Claim 2, characterized in that the cationic lipid is
 DMRIE.

4. Immunogenic preparation or vaccine according to
 25 Claim 3, characterized in that the DMRIE is coupled to
 a neutral lipid.

5. Immunogenic preparation or vaccine according to
 Claim 4, characterized in that the DMRIE is coupled to
 DOPE.

30 6. Immunogenic preparation or vaccine according to
 Claim 1, characterized in that the element capable of
 increasing the immune response comprises a carbomer as
 adjuvant.

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7. Immunogenic preparation or vaccine according to Claim 1, characterized in that the element capable of increasing the immune response comprises a porcine cytokine.
- 5 8. Immunogenic preparation or vaccine according to Claim 7, characterized in that the porcine cytokine is GM-CSF.
9. Immunogenic preparation or vaccine according to Claim 7 or 8, characterized in that it comprises a 10 plasmid encoding and expressing the porcine cytokine.
10. Immunogenic preparation or vaccine according to Claim 1, characterized in that the element capable of increasing the immune response comprises a porcine cytokine and a compound selected from the group 15 comprising DMRIE, DMRIE/DOPE and carbomer, as adjuvant.
11. Immunogenic preparation or vaccine according to any one of Claims 1 to 10, characterized in that it comprises a plasmid encoding and expressing another porcine immunogen.

Figure 1/5
Plasmid pJP109

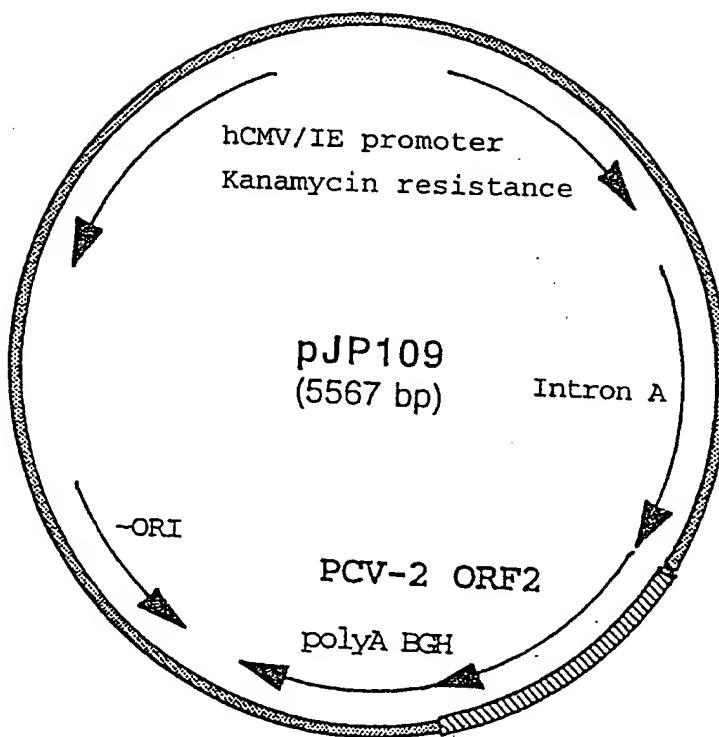


Figure 2/5
Plasmid pJP111

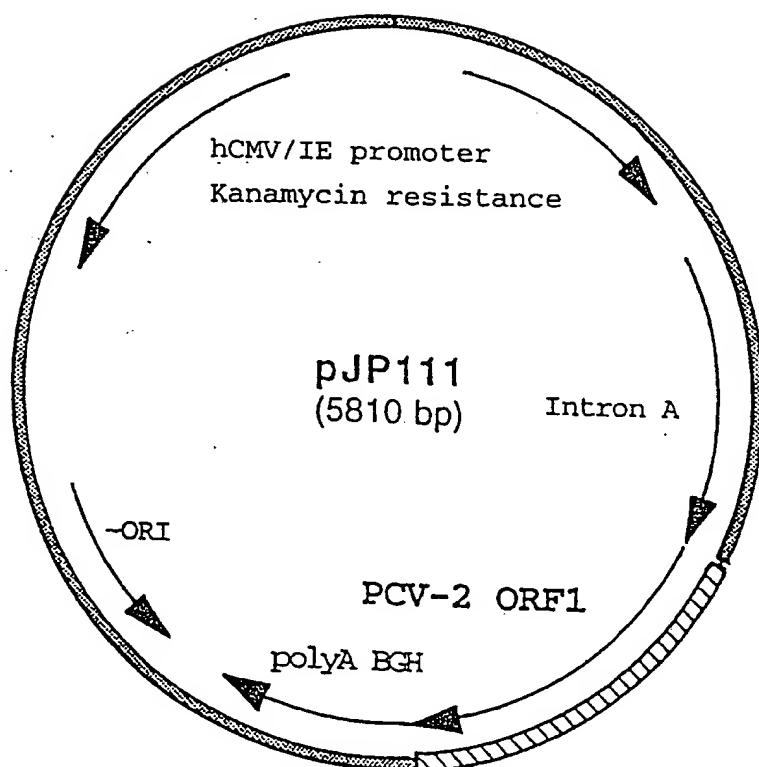


Figure 3/5
Plasmid pJP120

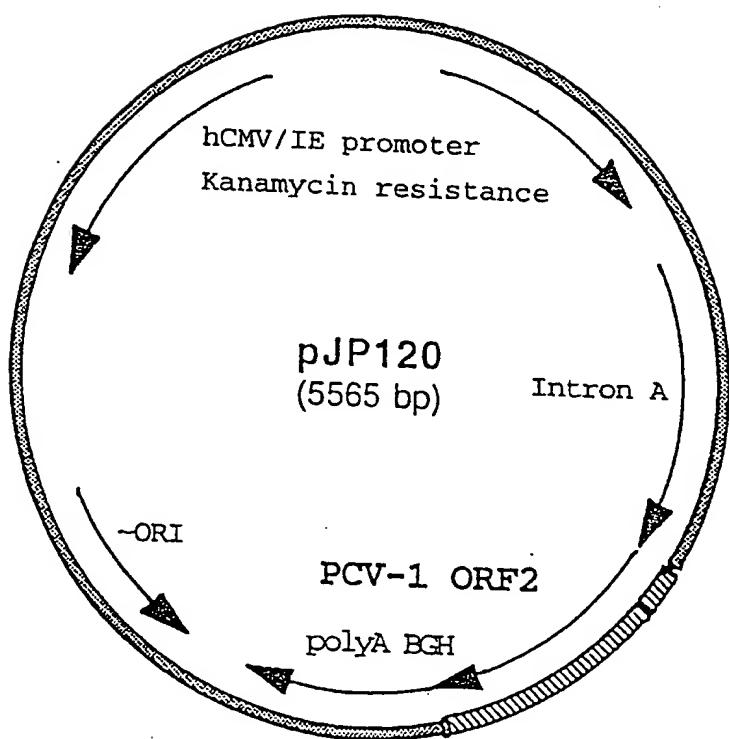


Figure 4/5
Plasmid pJP121

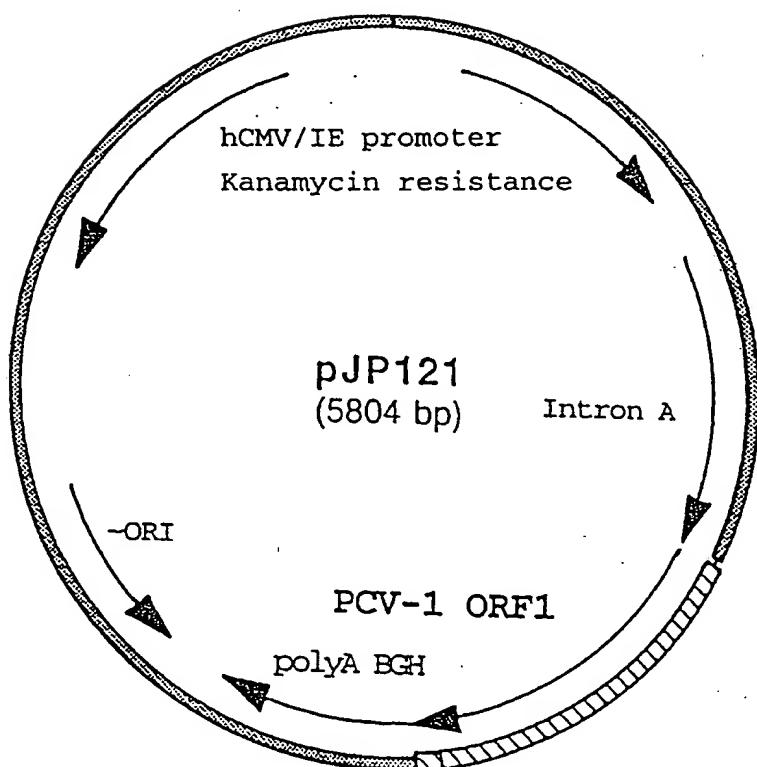
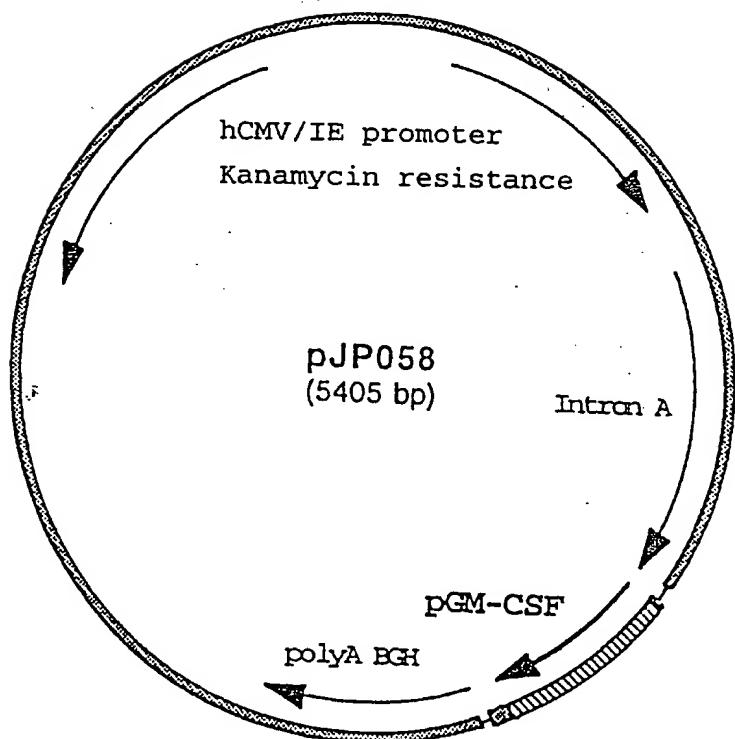


Figure 5/5
Plasmid pJP058



SEQUENCE LISTING

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<120> DNA vaccine - PCV

<130> DNA vaccine PCV

<140> numéro brevet

<141> date dépôt brevet

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<170> PatentIn Ver. 2.1

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34

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